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The Marek's disease virus (MDV) chemokine vIL8 binds the cCXCR5 receptor and is a functional orthologue of cCXCL13

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Dedicated

To my late father (Abu Nidal) who dreamt of seeing his son a doctor.

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List of Abbreviations

AcHV-	*Anatid herpesvirus-*
ADOL	The Avian Disease and Oncology Laboratory
ALV	Avian leukosis virus
APC	Antigen-presenting cell
BAC	Bacterial artificial chromosome
BEVS	The baculovirus expression system
BLC	B lymphocyte chemoattractan
bZIP	basic-region leucine zipper
CD	Clusters of differentiation
cDNA	Complementary deoxyribonucleic acid
CECS	Chicken embryo fibroblast
CGNC	The Chicken Gene Nomenclature Consortium
Chemokines	C**hemo**attractant cyto**kines**
CMV	Cytomegalovirus
CoHV	*Columbid herpesvirus*
ConA	Concanavalin A
CXCL	Cystine X (any amino acid) cystine Ligand chemokine
CXCR	Cystine X cystine receptor
DC	Dendritic cell
DMEM	Dulbecco's Minimal Essential Medium
DNA	Deoxyribonucleic acid
Ds	Double strand
EBV	Epstein-Barr virus
EtBr	Ethidium bromide
FBS or FCS	Fetal calf serum or Fetal Bovine Serum (FBS)
FC	fragment constant
FFE	Feather follicle epithelium
GAG	Glycosaminoglycans
GaHV	*Gallid herpesvirus*
gC	Glycoproteins C
GFP	Green fluorescent protein
HIV	Human Immunodeficiency Virus
HRP	Horse radish peroxidase
HSV	Herpes simplex virus
HVEM	herpes virus entry mediator

HVT	Herpesvirus of Turkey
I.E.	immediate early
ICP0	infected cell polypeptide number 0
ICTV	International Committee on Taxonomy of Viruses
IFN	Interferon
IL-	interleukin-
iNOS	inducible nitric oxide synthase
IRL	internal repeat long regions
Kbp	Kilo base pair
kDa	Kilodalton
LAT	Latency Associated Transcripts
LB	Luria-Bertani
LPS	Lipopolysaccharides
MD	Marek's disease
MDV	Marek's disease virus
MeHV	*Meleagrid herpesvirus*
MEQ	MDV EcoRI Q
MHC	Major histocompatability complex
miRNA	micro RNA
mRNA	Messenger ribonucleic acid
MSB1	Mesoblast
MWCO	Molecular weight cut-off
NAE	Non-Essential Amino acids
ng	Nanogram
NK	natural killer
NO	Nitric oxide
NPC	nuclear pore complex
NTC	no template control
O/N	Overnight
ORF	Open reading frame.
PAMPs	Pathogen associated molecular patterns
PBS	Phosphate buffered saline
pCMV	promoter for cytomegalovirus
PCR	Polymerase chain reaction
Pfu	plaque-forming units
PRRs	Pattern recognition receptors

RT	Reverse transcriptase
REV	Reticuloendotheliosis virus
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
RT	Room temperature
SQRT	Square root
TAE	Tris acetic acid EDTA
TCR+	T-cell-receptor-positive
TK	The Human Thymidine Kinase
TLR	Toll-like receptor
Tm	Melting temperature
TPBS	tween PBS
TRL	terminal repeat long
U_L	unique long
U_S	unique short region
vCK	Viral chemokine
vCKBP	Viral chemokine-binding protein
vCKR	Viral chemokine receptor
vv+MDV	very virulent plus strain
vvMDV	very virulent MDV strain

The Marek's disease virus (MDV) chemokine vIL8 binds the cCXCR5 receptor and is a functional orthologue of cCXCL13

Summary

Marek's disease virus (MDV) is a highly oncogenic *alphaherpesvirus* that causes Marek's disease (MD) in chickens. Infection of susceptible birds with virulent strain results in economic loss due to a high mortality, visceral lymphomas, paralysis and immunosuppression. The MDV genome encodes a CXC chemokine (vIL-8) that was named after the first identified chicken interlukin-8 (cCXCL8) based on the sequence similarity. However, recent functional data suggest that vIL-8 is not a cCXCL8 homologue. Identification of the cellular orthologue and receptor of vIL-8 are needed to understand its role in MDV pathogenesis. In the first part of this thesis, the evolutionary relationship was analyzed and vIL-8 functions characterized *in vitro*. Phylogenic and amino acid analysis of vIL-8 and all so far known human and chicken CXC chemokines revealed that vIL-8 lacks the conserved ELR (Glutamic acid- Leucine- Arginine) motif of CXC chemokines. The analysis also demonstrated vIL-8 has the highest sequence homology to cCXCL13L1 also known as B lymphocyte chemoattractant (BLC), which also lacks the ELR motif. Unlike the typical four exons structure of CXC chemokine, vIL-8 and CXCL13 have only three exons. In addition, we identified cCXCR5 as the receptor of vIL-8, which is the exclusive receptor of cCXCL13 and is present on chicken B and certain T cell subsets.

To address if cCXCL13 can complement vIL-8 in MDV pathogenesis and tumor formation, I used the bacterial artificial chromosome (BAC) system harboring the sequence of the very virulent MDV (vvMDV) RB-1B strain to generate recombinant viruses that express the cellular cCXCL13s in the absence of vIL-8. First, I inserted cCXCL13L1 downstream of vIL-8, but this locus did not allow secretion of cCXCL13. Next, I inserted the three variants of cCXCL13 (L1, L2 and L3) under the control of the immediate early cytomegalovirus promoter (pCMV) in the unique long region (at the C-terminus of UL35 and UL45), these recombinant mutants were unable to replicate *in vitro*. We then target the non-essential mini-F locus to insert CXCL13 driven by pCMV; however, the recombinant virus of CXCL13L1 was unable to replicate *in vitro*. Finally, insertion of cCXCL13 in mini-F driven by Thymidine kinase promoter (TK) allows reconstitution of recombinant viruses that secreted cCXCL13 and replicated comparable to wild type virus *in vitro.* We conduct *in vivo* experiment by infect one day chicks with the recombinant viruses and keep some as contacts. The recombinant viruses were replicate slightly lower that wild type in the infected animals, but unable to

tarnsmitte and replicate in contact birds. In addition, the mutants couldn't complement the loss of vIL-8 in MDV pathogenicity.

To explain the lack of complementation between vIL-8 and its cellular orthologue, I tagged vIL-8 with a polyhistidine-tag (6xHis-tag). This tagging enables comparable quantification of vIL-8 expression driven by its original promoter with TK-CXCL13 encoded in the mini-F. The results demonstrate that the expression level of vIL-8 is much higher than cCXCL13s driven by the TK promoter in mini-F, indicated that expression levels and kinetics are crucial for chemokine function.

1. Introduction

Marek`s disease virus (MDV) is a highly infectious herpesvirus that infects chickens and causes lymphomas. Marek`s disease (MD) is of high economic importance in poultry industries worldwide causing high mortality due to lymphoma and immunosuppression. MDV is a member of the *alphaherpesvirinae. Herpesviruses* have been shown to harbor genes with a high homology to cellular genes. These genes might be acquired from the host genome during long evolution. *Herpesviruses* utilize these genes and their products to maintain viral survival and enhance pathogenicity. Homologues genes are the gene with similar sequence and mostly perform a similar function. When these homologues gene acquired from the host during evolution, they defined as orthologues. Correct Identifying of such acquired viral genes and gene products is needed to understand its role, which may guide to the development of therapeutic strategies to prevent persistent viral infection.

One of these genes could be a viral chemokine named viral interleukin 8 (vIL-8) after initial similarity with chicken IL-8 (cCXCL8). However, functional similarity is not demonstrated, and the name of this chemokine is misleading. The aims of this project are to identify the true vIL-8 orthologues within host chemokine, and the main function(s) of this virokine. This characterization is of importance to understand gene function in both host and virus. In this section, background on MDV and the disease will be introduced before describing the particular objectives of the project.

1.1. *Herpesviruses*

The order *Herpesvirales* affected all organism`s levels from insects to human. At least one major disease of human and domestic animal species caused by a member of *Herpesvirales*. Important herpesvirus diseases include for example human cytomegalovirus (hCMV), herpes simplex virus (HSV), pseudorabies (Aujeszky's disease), infectious bovine rhinotracheitis (IBR), and Marek's disease in chicken (30).

Herpesviruses of human and animals have similar types of lesions, range from localized epithelial vesicular eruptions to diffuse damage in many organs (30). They also cause infection of the fetus which may lead to fetal death and subsequent abortion (121). An important feature of *herpesviruses* is that cause latency and tumor in their host. *Herpesvirus* of animal has a great concern particularly because of the economic losses they cause. Beside certain animal herpes infections used as model infections for the study of the human-related virus, which enable development of virus control strategies (112).

1.1.1. **Taxonomy and classification of *herpesvirus***

The last updated report released by the International Committee on Taxonomy of Viruses (ICTV), accommodates three families in the order *Herpesvirales; Alloherpesviridae*, which infects fish and reptile, *Malacoherpesviridae* infects mollusc species and *Herpesviridae* (62). The family *Herpesviridae* subdivided into one unassigned genus and three subfamilies: *Alphaherpesvirinae*, *Betaherpesvirinae,* and *Gammaherpesvirinae* based on virus structure, host range and the capability to establish latency in infected cells (62). *Alphaherpesviruses* have a wide host range, characterized by fast replication cycles and lifelong latent infection in neurons. *Betaherpesviruses*, in contrast, have a restricted host range and replicate more slowly than *alphaherpesviruses*. Infection with *Betaherpesviruses* often associated with cell enlargement (so named cytomegalovirus) and form latency in the monocyte series. Members of the subfamily *Gammaherpesvirinae* have a restricted host range, replicate and establish latent infection in lymphocytes (62).

Alphaherpesvirinae consists of five genera: 1) *Iltovirus*: affect only birds and includes only one species, gallid herpesvirus-1 that causes avian infectious laryngotracheitis. 2) *Mardiviruses:* affected only birds and associated with malignancy; which includes Marek's disease virus. 3) *Simplexvirus*: mainly affect primates and establish latency in neurons; for example herpes simplex virus1 (HSV-1) which causes cold sores in humans. 4) *Varicellovirus*: establish latency in the sensory nervous system; include varicella-zoster, the causative virus of chickenpox and shingles in humans. Finally, 5) *Scutavirus* and one unassigned genus, which cause respiratory infection in turtles (41, 119). The genus *Mardivirus* (*Mardi*: from **Mar**ek's **di**sease) includes three closely related but distinct species of Marek's disease viruses **(Table 1)**.

Gallid Herpesvirus2 (GaHV-2) or commonly known as MDV type1, contains all virulent strains (Md5, RB1B, SD2012-1 and others), as well as some attenuated strains such as CVI988/Rispens. GaHV-3 and *Melagrid Herpesvirus* 1 (MeHV-1) are both considered a mildly pathogenic and elicit antigenic cross-reactivity to GaHV-2, which has been exploited in vaccines. Virulent GaHV-2 strains were further classified by The Avian Disease and Oncology Laboratory (ADOL) as mildly virulent, virulent, very virulent and very virulent plus. This pathotype classification indicates the ability of the strain to break immunity and induce lymphoma in CVI988-vaccinated chickens compared to the MDV reference strains (144, 145).

Table 1: Nomenclature and classification of Marek`s disease virus

Marek's disease virus classification is mentioned by gray areas, and type-species are indicated in bold. Adapted from King et al.(62).

Order	Family	Sub-family	Genus	Species
Herpesvirales	*Alloherpesviridae*		*4 Genera*	
	Malacoherpesviridae		*2 Genera*	
	Herpesviridae	*Alphaherpesvirinae*	*Iltovirus*	*Gallid herpesvirus 1*
				Psittacid herpesvirus 1
			Mardivirus	*Anatid herpesvirus 1*
				Columbid herpesvirus 1
				Gallid herpesvirus 2 (MDV-1)
				Gallid herpesvirus 3 (MDV-2)
				Meleagrid herpesvirus 1 (MDV-3, HVT)
			Scutavirus	*Chelonid herpesvirus 5*
			Simplexvirus	*11 species*
			Varicellovirus	*17 Species*
		Betaherpesvirinae	*4 Genera*	
		Gammaherpesvirinae	*4 Genera*	
		Unassigned	*Unassigned*	*Iguanid herpesvirus 2*

1.1.2. Structure and genome of *Herpesvirus*

Herpesviruses virion are spherical to pleomorphic in shape and 150-200 nm in diameter **(Figure1):** The virion has a three-layered structure including a large dsDNA genome enclosed within an icosapentahedral capsid, together known as nucleocapsid which forms the core of the virus. The capsid has a portal to allow viral DNA in and outgoing movement during viral replication. Amorphous protein coat called tegument surround the capsid contain proteins that required for viral replication. The outer layer composed of glycoprotein spikes embedded in the lipid bilayer envelope, members of the same family in herpesviruses share antigens, but envelope glycoproteins are specific for each species (26).

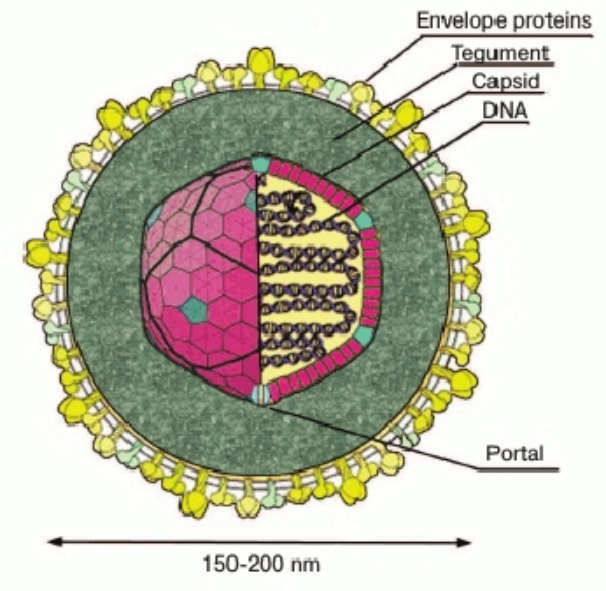

Figure 1: Schematic representation of Herpe s virion

The virion is spherical to pleomorphic, 150-200 nm in diameter. Capsid is icosahedral symmetry containing double stranded DNA genome, viral DNA enters and exits the capsid via the capsid portal. The capsid is surrounded by an amorphous tegument. Glycoproteins spikes are embedded in the lipid envelope. Obtained from Kukhanova et al. (68).

The genome of *Herpesviruses* is linear dsDNA, 125-295 kbp in size, encodes 70 to more than 200 proteins (62). It is constituted of a unique (U) and repeat (R) region sequences. The *Herpesviruses* DNA genomic arrangement can be divided into six groups, designated as A to F **(Figure 2)**. In the class A genome, the unique sequence flanked by direct terminal repeats, it is represented in human herpesvirus 6 of *Betaherpesvirinae*. Class B genomes have tandem direct repeated sequences at the both ends. Most of the *Gammaherpesvirinae* represents this arrangement. The structure of class C is almost like class B, but with extra internal direct repeats that unrelated to the terminal repeat as in Epstein-Barr virus (EBV), a member of the *Gammaherpesvirinae*. Class D genomes contain long and short unique regions (UL and US), flanked by inverted terminal repeats (TRL/IRL and TRS/IRS); this structure represented in Duck enteritis virus and *Varicellovirus* genus of *Alphaherpesvirinae* (12). Class E genome is related to class D but with two main differences; relatively larger TRL and IRL, and the presence of an a-like sequence bracketing the TR and separating the IRL and IRLS (64). Class E arrangement is represented in most of *Alphaherpesvirinae* members; *Simplexvirus* genus, HCMV, and MDV. Class F lacks the terminals and internal repeats that characterize other herpesvirus genomes; it represented by some members of the *Betaherpesvirinae* (12).

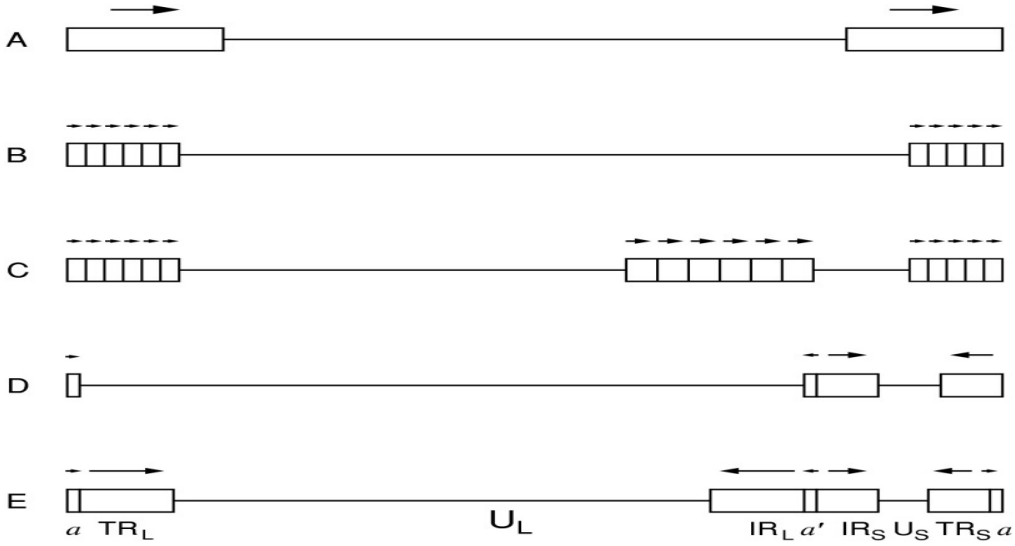

Figure 2: Classes of *herpesvirus* genome structures

Unique and repeated sequences are shown as solid lines and rectangles respectively. The repeated sequences flanking the unique regions are named TR_L, TR_S, IR_L and IR_S. The orientations of repeated sequences are indicated by arrows. U unique, L long, T terminal, I internal, S short, a- a-like sequences. Obtained from Arvin et al. (12)

1.1.3. Life cycle of herpesvirus

Recognition of virus life cycle is essential to understand its pathogenicity, as well as on the development of specific vaccines and therapeutics which interfere with virus replication steps to reduce disease progress. *Herpesvirus* life cycle begins with the interaction of virion envelope glycoproteins with target host cellular receptors **(Figure 3)**. Members of herpesvirus have different envelope glycoproteins which bind to different cellular receptors and have multiple functions, for that; this section will focus mainly on the *alphaherpesvirus*.

Among many *alphaherpesvirus* envelope glycoproteins, five glycoproteins have defined roles in viral entry through membrane fusion, which are glycoprotein B (gB), gC, gD, gH, and gL. *Alphaherpesvirus* gC are type 1 membrane proteins with a short transmembrane domain; it binds mainly to various of glycosaminoglycans (GAG) cell surface receptor includes heparin and chondroitin-like-GAG. Other spliced forms of gC proteins that lack the transmembrane domain make the majority of MDV gC in a secreted form *in vitro* (54). However, neither gC nor heparan sulfate is essential for viral entry into target cells since a gC knockout mutants can infect cells but with low efficiency (133). Glycoproteins B and D stabilized or can substitute gC in mediating binding (123). The gD binds to three classes of cell receptors:

herpes virus entry mediator (HVEM), nectin-1 and nectin-2 (142), forms a multi-protein fusion complex with gB, gD, and the heterodimer gH-gL, result in conformational change allowing to release of viral nucleocapsid into the cytoplasm (123). Some viral entry can also occur by low-pH-dependent and independent endocytosis depending on the virus and the cell type (67). Once the virus successfully entered into the target cell cytoplasm; capsid and some tegument proteins are docking at nuclear pore complex (NPC), undergo a conformational change, creating an opening to allow the release of viral DNA (uncoating) into the nucleus (65). The UL36 gene encodes tegument protein product VP1/2, which is conserved among all herpesvirus subfamilies. The VP1/2 have nuclear localization signal which plays an essential role in capsid transport and viral DNA entry into the nucleus (59). Once the DNA enters the nucleus, tugment proteins VP16 encoded by UL48 gene activates viral immediate-early (IE) genes transcription (α genes) (68).The UL41 gene encodes Virion Host Shutoff Protein (VHS) expressed with Immediate-early kinetics, which inhibits host cell protein synthesis and viral mRNA degradation (87). Expression of α genes mainly Infected Cell Proteins (ICP4 and ICP27) are required to inhibit cellular defense against virus and facilitates expression of early genes (β genes) (91, 109). DNA polymerase encoded by β gene UL30 is involved viral genome replication. Other β genes encode a variety of enzymes and proteins which are involved in modifying host cell metabolism, suppression of early α genes and activation of late genes (γ genes) (23).

The viral structural proteins including envelope glycoproteins and the capsid proteins are encoded primarily within γ genes (91); they synthesized in the cytoplasm and are translocated to the nucleus. The capsid is assembled with viral genome, providing of progeny virions. Nucleocapsid then egresses from the nucleus by crossing nuclear membrane pores, or by budding through the nuclear membrane into the cytoplasm. Final maturation involves assembly of tegument proteins and envelope glycoproteins; this step takes place through budding of virion into Golgi-derived vesicles. The vesicle membrane contains assembled virion then fused with the plasma membrane of the cell and the mature virion released (80).

Herpesviruses can infect target cells and maintain its genome without a full productive cycle, which described as the latent state of infection (Latency) (48). During latency, the virus can maintain its genome in different forms; either integrated within cell genome or in a circular form within cellular nucleus called episome (131).

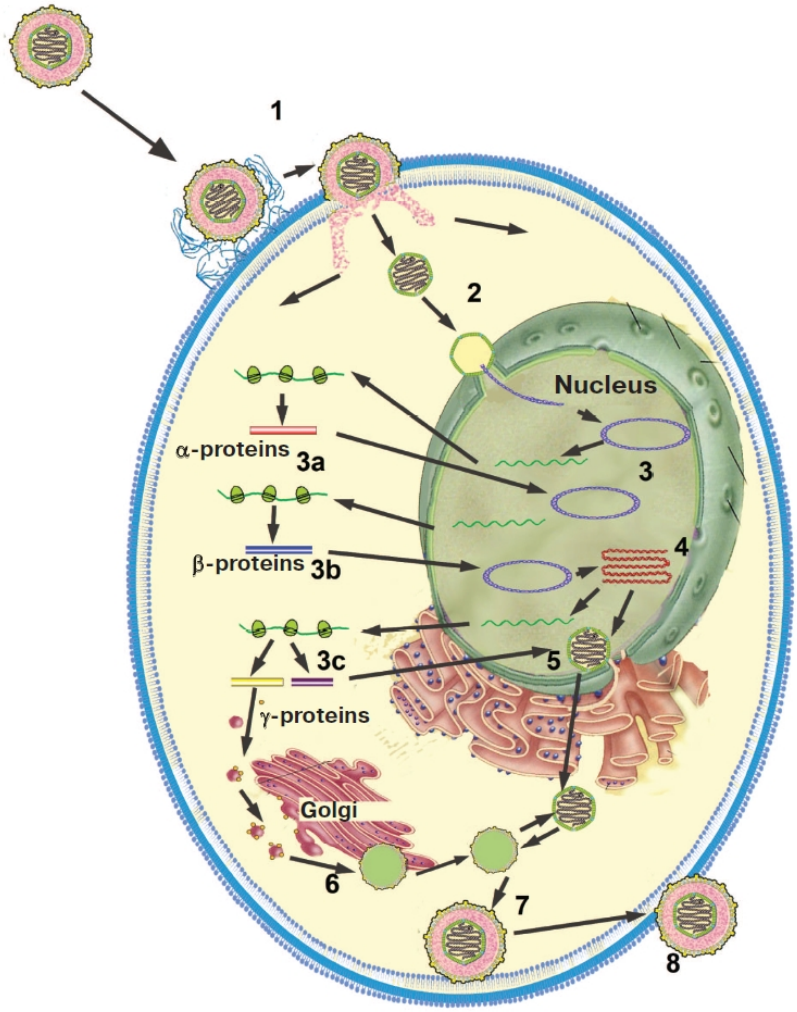

Figure 3: *Herpesvirus* productive life cycle

1) Virion glycoprotein attaches to cell membrane receptor; virion enters and release tugments proteins into the target cell cytoplasm. 2) Transport of nucleocapsid to the nucleus and uncoating. 3) Expression of viral gene: (a) immediate early, (b) early, and (c) late genes. 4) Viral DNA replication and nucleocapsid assembly. 5) Nuclear egress. 6) Capsid maturation. 7) Primary envelope formation. 8) Virion egress form cell membrane. Obtained from Kukhanova et al. (68).

In *Alphaherpesviruses* latency, only Latency Associated Transcripts (LAT) were expressed, while beta- and *gammaherpesviruses* express latency proteins. A low level of α and β genes expression can occur but is not sufficient to initiate a productive replication (49). LATs expression maintains latency by silence expression of IE genes through the existence of micro-RNA (miRNA); enable virus persistence by inhibition of cellular immunity and cellular apoptotic pathways. The LAT also required for reactivation of the virus (44); activation from latency is initiated by stress in the host cell, in which the virus return to cytolytic replication, forms cytopathic lesions and complete the life cycle (48).

1.2. Marek's Disease (MD)

1.2.1. Background

József Marek (1868–1952) was a Hungarian veterinary pathologist at the Royal Hungarian Veterinary School in Budapest; his name will be forever remembered due to his contributions in the field of virology. In 1907, he described a polyneuritis disease which affects wings and legs of adult chickens. After that, the disease was renamed to 'neurolymphomatosis gallinarum as the lesions were not only restricted to the nervous system, but also lymphatic tumors were distributed in different visceral organs (96). After the discovery of the causative agent in 1967, the disease was named Marek`s Disease (MD) in the honor of József Marek (118). Chickens are the main target species of MD; also, it can infect other species such as turkeys, quail, and pheasant (99). MD is a highly fatal lymphoproliferative tumors in susceptible chickens and causes a high economic losses on the global poultry industry (82). It became the first and most important disease model in which a neoplastic condition was successfully controlled by vaccination (62, 112). The natural route of MD infection is through inhalation of cell-free virus particles by chickens. Susceptible birds develop clinical signs such as depression, paralysis, immune suppression, and lymphomas in different organs. The severity of MD is varied, depending on the host genetic susceptibility, vaccination status and the virulence of MDV strain (112). Vaccination and efficient management practices significantly reduced the losses caused by MD. Non-pathogenic strains of MDV-1, MDV-2, and MDV3 individually or in combination have been successfully used against MD (86). Afterward, new field strains of pathological MDV classified as very virulent (vvMDV), and very virulent plus (vv+MDV) were isolated (144). These virulent strains are characterized by earlier mortality, accompanied with increase lymphoma affecting broad tissue tropism, and immunosuppression (18). The emergence of highly virulent MDV field strains after extensive use of vaccines (vaccine breaks) may be related to effective selection pressure of MDV, which supports the hypothesis of continued evolution of MDV towards a greater virulence (144). This race between vaccination and vaccine breaks requests more studies on virus gene function as well as virus-host interaction to develop an efficient and endurable vaccine that prevents MDV vaccine breaks (39). The development bacterial artificial chromosomes (BAC) and its application to MDV genome enhance the characterization and manipulations of virulence genes in MDV (86). Also, the release of the whole chicken genome sequence allows the opportunity to understand the genes involved in MDV pathogenesis and genetic resistance to MD (86).

1.2.2. Clinical signsof MD

Marek's disease is a progressive disease with several overlapping signs and pathological syndromes. Susceptible chickens infected with MD show specific and nonspecific symptoms. The nonspecific symptoms include pallor, weight loss due to inability to reach food and water (148). Specific clinical MD signs include different overlapping syndromes with variable signs which are described as classical MD (Fowl Paralysis), acute MD (Transient paralysis), Ocular Lymphomatosis, cutaneous MD, and immunosuppression (77).

Classical MD (Neurolymphomatosis or so-called Fowl Paralysis) represent clinically by asymmetric paralysis of one or both legs due to infiltration of lymphomas in brachial and lumbosacral nerves **(Figure 4A)**. Affected nerves appear gray to yellow due to inflammation and lymphocyte infiltration with an increase in their normal size. Incoordination due unilateral paresis or paralysis is a common early sign, characterized by one leg held forward and the other backward **(Figure 4A)** (99). Dilation of the muscular stomach and gasping can be found if infiltration involves the vagus nerve. Lymphoma infiltration also occurs in visceral organs such as the spleen, kidneys, gonads, skeletal muscle, proventriculus and liver **(Figure 4B)** (148)

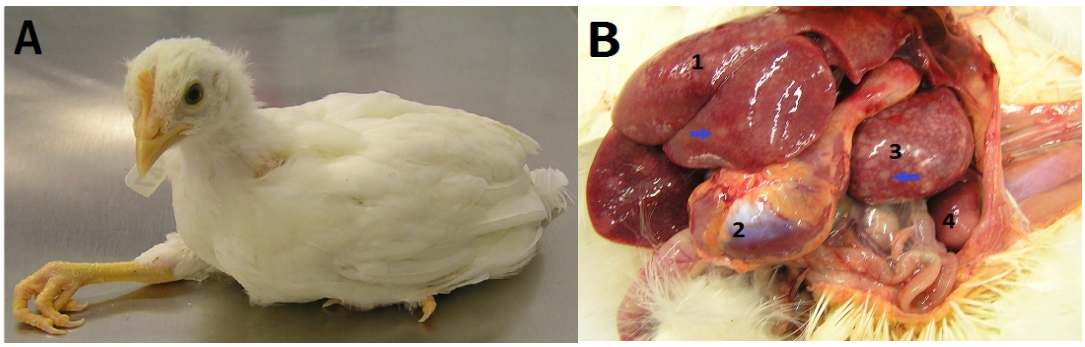

Figure 4: Clinical picture and necropsy findings of MD
(**A**) Unilateral leg paralysis caused by lymphocytic infiltration into the peripheral nerves. (**B**) (**1**) enlarged liver with white nodular lymphomas (arrow) scattered throughout the organ.(**2**) muscular stomach (gizzard) swelling.(**3**) A lot of white diffused lymphomas (arrow) scatted in the swelled spleen; (**4**) kidney swelling and pale in color.

Acute MD occurs in sudden outbreaks with the neck and limb paralysis was observed in some infected chickens. Post-mortem lesions include various degrees of edema due to inflammation of the cerebrum, cerebellum, and brain stem. Lymphomas of many visceral

organs described in acute MD, commonly occur in the spleen, kidney, liver, gonads, proventriculus, thymus and skeletal muscle (16).

Transit paralysis caused by vvMDV or vv+ MDV strains and characterized by the onset of neurologic signs 9-10 days post-infection (dpi); symptoms related to brain lesions and include flaccid paralysis of neck and limbs. Death typically occurs 1-3 days after the onset of clinical signs with few if any gross tissue changes that can be found (146). Ocular lymphomatosis form caused by infiltration of transformed cell in one or both eye; affected eye(s) appear with irregular gray iris and eccentric pupil, consequently partial or total blindness may happen (95). Cutaneous Marek's disease is recognized readily after plucking of feathers, round nodular lesions up to 1 cm in diameter are seen particularly around feather follicles (34). Immunosuppression form of MD includes both humoral and cellular host immune defense arsenals; the early cytolytic infection of B lymphocytes, latent infection of both B and T lymphocytes, and late cytolytic infection and transformation of T lymphocytes results in severing immunosuppression state. Lymphoid organs targeted by MD lesions include the source of chicken B and T lymphocyte (bursa of Fabricius and thymus respectively), as well as spleen (site for immunological recognition) (52). Affected birds become more susceptible to secondary infections mainly *Escherichia coli.* (52). Morbidity and Mortality rates for MDV virulent strain can reach up to 100%in unvaccinated birds (82).

1.3. Marek`s Disease Virus (MDV)

1.3.1. MDV genome

The genome of MDV represents E class arrangement of *Herpesviridae* **(Figure 2)**. It is a linear double-stranded DNA, of about 178 kbp in size. It comprises two unique regions long (U_L) and short (U_S), separated by inverted repeats long (IR_L) and short (IR_S), and flanked by the terminal repeat long (TR_L) and short (TR_S) (118). The a-like sequence is flanking the TR and separating the IR_L and IR_S. The TR_L and IR_L, as well as the TR_S and IR_S, are identical in sequence and present in inverted orientations (91). The genes located in the unique long region are identified as homologs in members of the *Alphaherpesvirinae* (36), whereas genus and virus-specific genes encoded by MDV, GaHV-3, and HVT found in the inverted repeat regions (118). MDV genome encodes more than 100 ORF and proteins, viral virulence and oncogenicity are differing from strain to strain, based on specific genes observed in each species. For example, oncogenic MDV-1-specific meq gene (**M**DV **E**coRI-**Q**) has an essential role in tumor formation (25). Other MDV-1 strain-specific gene is a viral CXC chemokine encoded by two genes copies (MDV003 and MDV078 located in IRL and TR respectively), previously described as an IL-8 homolog (98); however, IL-8 gene and protein structure, as well as activity, was not demonstrated (75). On the other hand, MDV-3

unique gene is an NR-13 cellular homolog, encodes a putative Bcl homolog that regulates apoptosis (63).

1.3.2. MDV pathogenesis

Infection with MD begins with the inhalation of cell-free virus that was shed by infected chickens to the respiratory system. For best understanding of the sequential changes during pathogenic MDV infection; Cornell model reviewed by Calnek and colleagues provides a relevant description and is still accepted (32). According to Cornell model, MDV pathogenesis divided into four phases: (i) early cytolytic (2-7 dpi) (ii) latent phase (7-10 dpi), (iii) late cytolytic and immunosuppressive phase (18 dpi) and (iv) transformation phase (28 dpi) **(Figure 5)**.

1.3.2.1. Infection and early cytolytic phase

MDV infects and establishes a progressive, fatal disease in chickens. The natural route of infection is through inhalation of cell-free virus particles existing in virus-contaminated dust and feather dander. Dripping of feather tip extract to the eye and mouth of the birds can also induce infection experimentally through mucosal route. (33). Virus particles may infect parenchymal cells of the lung or taken up by phagocytic alveolar cells mainly macrophages and dendritic cells. These cells transmit the virus via lymph or blood to various lymphoid tissues; such as thymus, spleen, cecal tonsils and bursa (3), evidence of virus replication was also found in the phagocytic cells **(Figure 5)** (19).

During the early phase, the secreted virokine vIL-8 plays a significant role as a chemoattractant for B cells as the target cells for cytolytic infection (37, 43). Infected B cells can be detected in the lung as early as two dpi, which may come from circulation or directly infected in the lung (13). Initial cytolytic infection occurs in B cells resulting in the first phase of lympholysis and semi-productive viral replication (naked virion) without the release of cell-free virus (39), and virus spreading requires direct cell to cell contact. Cytolytic replication in B cells leads to suppression of humoral immunity. CD4+ T cell is the target cell where the virus establishes transformation and a latent infection (14), they become activated and then infected by direct contact with infected B cell (107), or may also become infected directly from macrophages without requiring activation by contact with infected B cells (13). CD4+ CD25+ Treg cells recruited to the site of infection by the chemoattractant effect of vIL-8, these cells might have a role in suppressing host immune responses starting the induction of the latent phase (43). The pp38 is necessary to induce cytolytic in B cell but not required during the late cytolytic replication in feather follicle epithelium or for horizontal transmission of the virus (46). Cytolytic phase is not essential for initiation of latent and transformation

phases and lymphoma formation; removal of all B cells by embryonal bursectomy before MDV infection or abrogation of vIL-8 recruitment of B cell reduced MDV virulence but didn't prevent latent infection or transformation (37, 113).

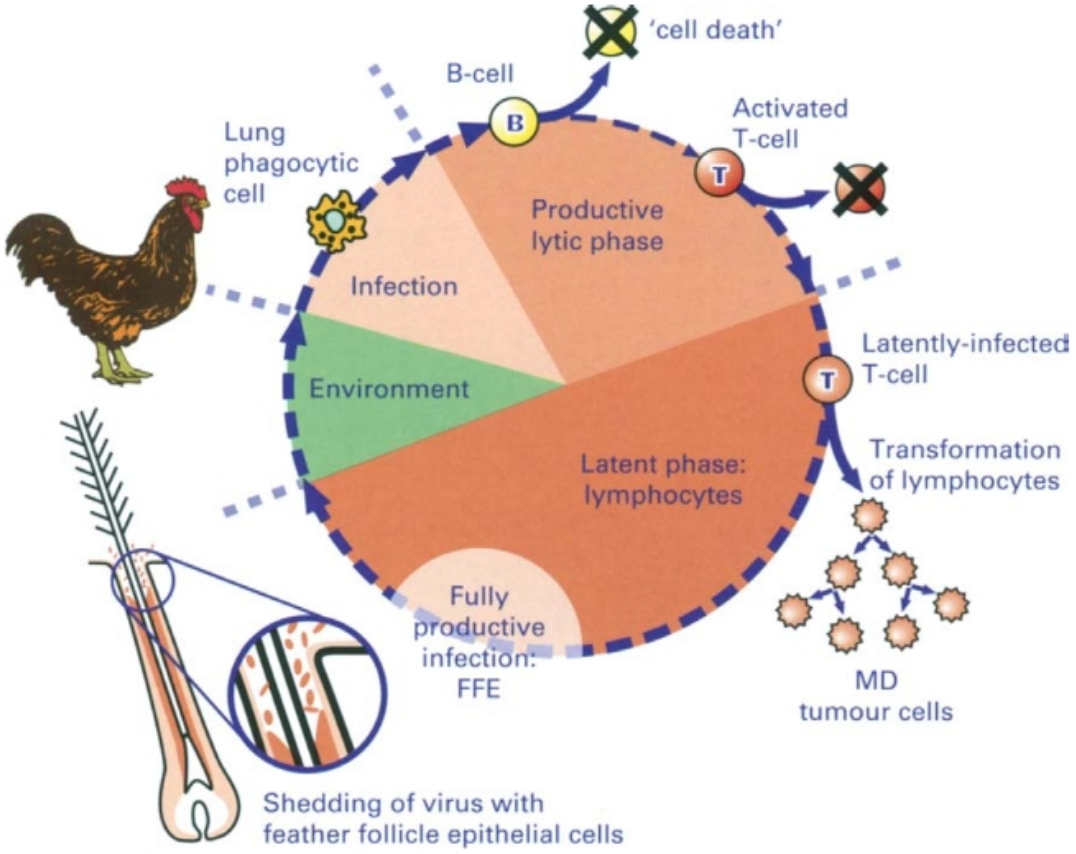

Figure 5: Cornell Model for MDV pathogenesis

Schematic drawing depicting different stages in the cycle of MD pathogenesis based on Cornel Model. More details can be found in the text. Obtained from Baigent et al (16)

1.3.2.2. Latent phase

MDV establishes latent infection in primarily CD4+ T lymphocyte. The latent phase starts when the host immune response initiated around seven dpi (3). During latency, viral genome can be detected but with minimal expression of viral or tumor antigens. The virus expresses some genes such as latency-associated transcripts (LATs), which are spliced RNA play a role in the balance between latent and lytic infections by interfering with translation of the immediate early(I.E.)regulatory protein (87). The meq gene has been shown to maintenance latency by activating latent gene expression and block apoptosis of latently infected CD4+ T cells. It activates also promoters of transcripts associated with oncogenicity (97).

Host immune status has a crucial role in the establishment, maintenance of latency and reactivation to cytolytic infection. Immunocompetent or resistance birds can develop clinical latency without clinical signs (38). In contrast immunocompromised chickens either by thymectomy or chemically induced immunosuppressive treatments exhibit a prolonged and more widespread early cytolytic phase after MDV infection (31). Immunosuppression treatment after latency (14 dpi) resulted in reactivation of a second cytolytic infection (31). This is consistency with immunosuppression accompanied MDV to reactivate a second productive viral cycle after latency (31). The latently infected T cells serve as reservoirs of the virus, migrate to distribute the virus to the target organs via the lymph and bloodstream. These cells can also undergo neoplastic transformation and proliferate to form gross lymphomas in susceptible birds (16).

1.3.2.3. Late cytolytic and immunosuppressive phase

Lymphocyte depletion due to lympholysis during early cytolytic phase results in inhibition of both humoral and cellular-mediated immune response. Immunosuppression state is a significant feature of MDV infection and occurs around 18 dpi onwards (16), which reactivates disseminated latently infected lymphocytes mainly T cells, results in the severe late cytolytic infection (16). Late cytolytic phase in T cells is characterized by productive replication in the feather follicle epithelium results in high levels of viral replication and infectious cell-free viral particles.These particles are shed in feather dust throughout the life of an infected bird which continues the viral life cycle (16). Upon immunosuppression, latently infected T cells can also proliferate to form gross lymphomas, responsible for the symptoms and eventual mortality of MD (16).

1.3.2.4. Transformation

Latently infected lymphocytes are disseminated during the early and latent phase into different visceral and nerve tissues; these cells can reactivate and undergo neoplastic transformation to form lymphoma around 3-4 weeks after infection **(Figure 5)** (97).The CD4+ CD8- T-helper cell is the primary target cells for MDV transformation (28). The main feature of neoplastically transformed cells are CD4+, major histocompatibility complex (MHC) class I, MHC class II hi, αβ T-cell-receptor-positive (TCR+), CD30 hi and other (28, 29). MD lymphoma matches with the neoplastic transformation criteria; as they have a high uncontrolled proliferation rate, they are protected from cell death, transformed from latency not productively infected, present in all MD lymphomas, and they have immune escape mechanisms to survive as a tumor cell (110). Several factors encoded in the MDV genome are involved in the establishment and the maintenance of tumorigenesis; for instance: phosphoprotein 38 (pp38), ICP4, viral telomerase RNA, vIL-8, and Meq were characterized

in MDV tumorigenesis (147). Uncharacterized genes may also play a role in tumorigenesis. The meq gene is the most described candidate among these genes (127), it was named after its location in MDV genome (**M**arek's **E**coRI **Q** fragment). MDV genome encodes two copies of meq gene (MDV005 and MDV076) within IRL and TRL regions respectively. Meq gene encodes 339 amino acid nuclear phosphoprotein (MEQ), and shares significant homology with the host basic-leucine zipper (bZIP) transcription factors Jun/Fos family (147). The structure of MEQ protein also includes a nuclear localization signal and a proline-rich transactivator.This structure enables MEQ protein to translocate to the nucleus and to dimerize with itself or with a variety of host bZIP partners including c-Jun, B-Jun, c-Fos, and CREB, and can functionally substitute host proto-oncogene (127). Also, it can regulate specific genes associated with cell proliferation such as CDK2 (76). Furthermore, MEQ is capable of inhibiting apoptosis (74). These unique features may provide meq gene as a major oncogene in MDV pathogenesis in establishing latency and cellular transformation.

pp38 is the product of MDV074 also has a role in maintaining both latency in T cell and the transformation *in vivo* by blocking apoptosis (46). The MDV-ICP4 gene (MDV084) within the Rs region has a crucial role in the regulation of transcription of many early and late genes. The ICP4 and its related transcripts have been detected at high levels in latently infected cells. The inactivation of ICP4 protein expression with antisense oligonucleotides inhibits proliferation of lymphoblastoid cells implies ICP4 and its related transcripts role in latency and transformation (33). The specific mechanism of the ICP4 in latency and transformation is not completely understood (147). Interference with the expression of ICP4, pp38, or MEQ proteins resulted in alteration of the transformed phenotype of Marek's disease virus-transformed lymphoblastoid cell line MSB1; assumes that these genes have essential in the maintenance of latency and transformation of MDV pathogenesis (147).

1.3.3. **Role of viral CXC (vIL-8) in MDV pathogenesis and tumorigenesis**

MDV encodes two copies of a CXC chemokine in the IRL and TRL regions (MDV003 and MDV0078 respectively), named viral interleukin-8 (vIL-8) (93, 98). The structure of vIL-8 gene consists of three exons and two introns. Exon I encodes the signal peptide of 22 amino acid, cleavage of exon I allows secretion of exons II and III which form the putative virokine (61). Besides, the splice variant of exons II and III is the acceptor site for the DNA binding domain of the oncogene meq and other upstream ORFs. The MEQ/vIL8 is nuclear localization oncoprotein, dimerize with either meq or c-Jun and binds to the activator protein 1 (AP-1) as a heterodimer (9, 56). Deletion of the entire vIL-8 ORF resulted in attenuation of the virus and induction of significantly less gross tumor development. However, this deletion

was also resulted in delays meq expression and impairs pp38 expressions in lymphoid organs(36), indicate the importance of exons II and III of vIL-8 to Meq and other upstream genes, including RLORF4 and RLORF5a (56).

As mentioned earlier, secreted vIL-8 involved in the chemoattraction of target cells to the initial infection site at the early phase of infection. vIL-8 attracts B cells as the target for lytic infection, as well as CD4+ CD25+ T-cells for latent infection and transformation (43). Complete deletion of vIL-8 gene resulted in mildly virulent virus (decrease in clinical signs, gross lesions, and mortality). Still, this deletion was also found to interfere with the role of pp38, meq, or may other close genes (36). Recently, it has been shown that the abrogation of vIL-8 expression (without deletion or interfering of splicing with other related genes) reduces mortality and tumor formation, suggesting the role of the secreted virokine in the pathogenicity and tumor formation of MDV (43). However, the mechanism mediates vIL-8 function and the target receptor(s) need further investigation.

1.4. Chemokines and chemokine receptors

Upon virus entrance to the respiratory tract, pathogen recognition receptors (PRRs) on the surface of various cells detect conserved virus pathogen-associated molecular patterns (PAMPs) (50, 58). Many genes such as chemokines are induced following PAMPs-PRRs binding to coordinate leukocytes function during host defense response. These chemokines (a shortening of **chemo**attractant cyto**kines**) are families of small (6–14 kDa) secreted proteins, which induce migration of leukocyte to the site of infection or initiate immune responses (117). In addition, they have been shown recently to perform a variety of functions in biological processes, such as lymphoid organ development (10), hematopoiesis (27), homeostasis (70), wound healing, atherosclerosis, and tumorigenesis (69, 104, 122).

Chemokines act by binding to seven transmembrane type-1 G-coupled receptors on the surface of cells, increase intracellular calcium influx and induce a cellular response. The receptors can bind only one or several members of the same class of chemokines (15). Chemokine receptors are expressed on many cells, for instance, endothelial cells (124), APC, and lymphoid cell (84).

The chemokines-chemokines receptors interaction evolved to benefit the host. However, viruses have developed different strategies to take advantage of these protein interactions to enhance their virulence and pathogenicity. For instance, some viruses use the chemokine receptors as a portal for virus entry (83). Other viruses encode chemokines that have an anti-viral effect (4), or secrete a viral chemokine (virokine) with functional homology to host

chemokines to recruit target cells to the site of infection (98). Moreover, some viruses express viral chemokine binding proteins (vCKBP) that bind and neutralize host chemokine activity (139). Therefore, analyses of the role of these virokines are required to understand their role in virus-induced diseases, and to develop therapeutics or vaccines to control viral infection.

1.4.1. **Chemokine: classification and nomenclature**

Since their discovery, chemokines were named without a particular system of terminology. Most of their names representing their functions, e.g., macrophage chemotactic proteins, others named according to the source of production, e.g., breast and kidney-expressed chemokine (BRAK) and platelet factor 4 (PF4), other listed with interleukins (e.g., IL-8), or even named randomly such as the growth-regulated gene product (GRO) (89). This blurred taxonomy has caused significant confusion in identifying members of chemokine families associated with their particular receptor on the target cell, as well as their biological function. Currently, the International Union of Immunological Studies/World Health Organization (IUIS/WHO) Subcommittee on Chemokine Nomenclature recommend a new classification system for chemokines and chemokine receptor, classified chemokines into subfamilies based on the arrangement of the four conserved N-terminal cysteine residues (15, 89, 90). The new subfamilies are CC, CXC, CX3C, and XC, with the postfix L for ligand or R for receptors followed by a number to identify individual members **(Figure 6)**. Based on this classification, CCL and CCR are used for the ligands and receptors respectively when the first two cysteines in the chemokine are adjacent, CXCL and CXCR separated by one amino acid, CX3CL and CX3CR when separated by three amino acids, whereas the XC and XCR when one of the first two cysteines is missing (85). The other two cysteines residues preserve the proper folding of these molecules by forming two disulfide bonds with the first conserved cysteines residues. Under this nomenclature, for instance, GRO, PF4, IL-8, and BRAK became CXCL1, CXCL4, CXCL8, and CXCL14 respectively (89).

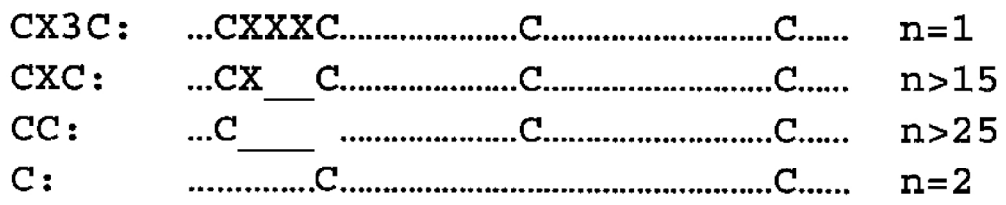

Figure 6: Structural classification of the chemokine families by signature cysteines
The number of members of each subclass in human is listed at the right of each structure. Numbers in chicken are (1 CX3CL, 8 CXCL, 14 CCL, and 1 XCL) Underlines indicate gaps in the alignment; X is an amino acid other than cysteine; and dots are other amino acids. The N and C termini can vary in length. Obtained from Murphy et al. (85).

At the functional level, chemokine can be divided into inflammatory or homeostatic.Inflammatory chemokines response to inflammation and tissue injury through induces chemoattraction of neutrophils, degranulation, angiogenesis, and vasodilatation. Homeostatic chemokines, in contrast, are expressed to regulate the migration of progenitor stem cells and leukocytes to the lymphoid organs. They also have an important role in organogenesis and embryonic developments (8, 150).

1.4.1.1. Chicken`s CXC chemokines

The release of chicken draft genome sequence by National Institutes of Health-University of California at Santa Cruz (UCSC) (2), and the comparative genomics studies of the sequence conservation between chicken and human offers exceptional opportunities for systematic identification and annotation of chicken chemokine and their receptor gene, and name them according to their orthologue in human and mouse genes (61, 141). Several genes of chicken and avian viruses encoding chemokines have been identified. This identification was based on the genes and amino acids syntenies, similarity and phylogenetic trees with related genes in human and mouse (141). Up to date, there are at least 14 putative chemokine receptors (4CXCR, 8CCR, 1XCR, 1CX3CR) and 23 putative chemokine genes (8 CXCL, 14 CCL, 1 XCL and 1 CX3CL) annotated in the chicken genome, representing all four classes of chemokines (61, 141). The majority of chemokine are within the CC and CXC group. As vIL-8 structure belongs to CXC chemokine, I will further focus on CXC chemokine.

Based on the conserved motif preceding the first cysteine; CXC chemokines can be divided into two subfamilies, (i) inflammatory (ELR+) CXC chemokine when an ELR (Glutamic acid-Leucine- Arginine) is present, (ii) whereas homeostatic (ELR-) are those where ELR is not found. ELR+ CXC chemokine regulates binding to CXCR1 and CXCR2 receptors, mediates both endothelial cell chemotactic and proliferation, induce activation and migration of

neutrophils (141). Also, they have a potential angiogenic effect. In contrast, the ELR- CXC chemokines bind to their conjugate receptors and almost exclusively attracts cells other than neutrophils, and has angiostatic properties (126).

CXC chemokine genes have a typical four-exon gene structure (except for CXCL13 which have three exons) (61). Eight CXC chemokines genes have been identified in chicken; three inflammatory (ELR+) and five homeostatic (ELR-) **(Table 2)**. Chromosome 4 encodes the three ELR+ CXC chemokine genes,the chemotactic and angiogenic factor (CAF), and K60. Both are the two previously described genes encode chicken orthologues to human CXCL8. However, more recently it was proposed that these chemokines to be called the chicken CXC inflammatory ligand (cCXCLi1 and cCXCLi2). The cCXCL1 is the third ELR+ CXC chemokine and it is proposed to named CXCLi3 (61).

On the other hand, homeostatic CXC chemokines distributed in three chromosomes; two are the orthologue to mammalian Stromal-derived factor-1 (SDF-1) or CXCL12, and breast and kidney-expressed chemokine (BRAK) or CXCL14, encoded by chromosome 6 and 13 respectively. Both CXCL12 and CXCL14 have the typical four-exon gene structures of CXC chemokine. The other three homeostatic CXC chemokines located on chromosome 4 and encodes three related genes; CXCL13L1, CXCL13L2, and CXCL13L3 with three exons gene structure (47, 61, 106, 141). CXCL12 binds to CXCR4 and CXCL12/CXCR4 pathway has a role in hematopoiesis and embryonic organs development, as well as B cell development and recruitment to the bursal mesenchyme (47, 105). CXCL14 is related to CXCL12/CXCR4 axis in embryonic development; however, its conjugate receptor and its role during embryogenesis remains unknown (47). CXCL13 also called B lymphocyte chemoattractant (BLC), the gene present only as a single-copy gene in mammals while the three related genes in chicken represent an expansion of this gene in avian species. CXCL13 in mammals binds CXCR5 on B cell has a role in lymphoid organs development (11).

Table 2: Chicken CXC chemokines and receptors

Classification of vIL-8 and chicken CXC chemokines based on the presences of ELR (Glutamic acid- Leucine- Arginine) motif and their receptors on target cells with main biological function involved in angiogenesis and associated with inflammation.

Sub-Family	Systematic Name	Classic Name	Major Receptors	Target Cell	Effect	Ref
CXC ELR+	CXCL1	CXCLi3 GRO-α	ND	ND	Inf	Kaiser et al. (61)
	CXCL8L1	CXCLi1 K60,	CXCR1, CXCR2	He, Gr, Mo	Inf, Ang	Poh et al. (102)
	CXCL8L2	CXCLi2 , CAF	CXCR1, CXCR2	He, Gr, Mo	Inf, Ang	
CXC ELR-	CXCL12	SDF-1	CXCR4,	B, T	Angio	Read et al. (106)
	CXCL13L1 CXCL13L2 CXCL13L3	BLC	CXCR5	B, T	Ans	Kaiser et al. (61)
	CXCL14	BRAK	ND	ND	ND	Gordon et al.(47)
CXC ELR-	vCXCL?	vIL-8	ND	B, T	ND	Engel et al. (43)

Ang: angiogenic, Ans: angiostatic, ELR (Glutamic acid- Leucine- Arginine) motif, He: heterophils, Inf: inflammatory, Gr: granulocyte, Mo: monocyte, B, T: lymphocyte, ND: not determined.

1.4.1.2. **Chicken CXCL8 (cIL8)**

Chicken chemotactic and angiogenic factor (cCAF), and cK60 are the two previously described genes encode the chicken orthologue to human CXCL8. As for human CXCL8, these genes located on chromosome 4 and encoded by a four-exon gene structure (51, 60, 78). More recently it was proposed these chemokines to be called the chicken **CXC i**nflammatory **l**igand cCXCLi1 and cCXCLi2 (61). However, the systematic nomenclature provided by the Chicken Gene Nomenclature Consortium (CGNC) is IL8L1 and IL8L2 for cCAF and cK60 respectively. As IL8 was renamed by IUIS/WHO/ Subcommittee on Chemokine Nomenclature to CXCL8, these genes will be named in this study as cCXCL8L1 for cCAF and cCXCL8L2 for cK60. cCXCL8L1(cCAF) the product of 9E3/CEF4 gene was the first non-mammalian cytokine cDNA to be cloned (128), it has been described as the chicken homolog of human CXCL8 (17). cCXCL8L1 and cCXCL8L2 proteins have 51% and 48% amino acid identity with human CXCL8. As for their orthologue; both chemokines are

members of the ELR+ CXC chemokine subfamily. The ELR motif determines receptor specificity to the neutrophils chemotactic receptors cCXCR1 and cCXCR2. cCXCL8L2 (cK60) is more efficient than CXCL8L1 in inducing the migration of monocytes while cCXCL8L1 is more efficient for migration of heterophils. Both are potent angiogenic and can initiate the wound-healing cascade *in vivo* (45, 78). Angiogenesis characterized by the neoformation of blood vessels, which is an essential biological event in the embryonic development, as well as in pathological processes, such as infection, wound healing and the growth and metastasis of tumors (45, 79).

1.4.1.3. **Chicken CXCL13**

In mammals, CXCL13 (or BLC) and its exclusive receptor CXCR5 are a key regulator of lymphoid tissue development. B cell and activated T-cell homing to lymphoid follicles requires CXCL13/CXCR pathway (71). T cells directed to enter the lymphoid follicles under the effect of CXCL13 after acquiring CXCR5 by direct contact with MHCII on APC. Activated T cells in follicle have a helper function to B cells and then called follicular B helper T cells (TFH),some also re-circulate as memory cells (24). CXCL13 knockout mice fail to develop lymph nodes (137). In contrast, the CXCL13/CXCR5 has implicated in the formation of ectopic lymphoid tissue during chronic inflammation and chronic bacterial infections (143).

Chickens have three adjacent CXCL13 genes on chromosome 4 named CXCL13L1, CXCL13L2, and CXCL13L3; all show identity and conserved synteny with the single the CXCL13 gene in human and mouse. Unlike the typical four exons structure of CXC chemokine; CXCL13 genes have a three-exon gene structure. At the functional level, CXCL13 classified within ELR- homeostatic CXC chemokines (61, 141), previously it was designated as cCXCL DT40 because it induces chemoattraction of chicken B cell line DT40 (125).The CXCL13/CXCR5 axis might be responsible for the migration of B cells into bursal follicles (105). High levels of cCXCL13 mRNA expression were found in acute and chronic *Mycoplasma* infection with high infiltration of CD4+ and CD8+ T-cells in affected organs, mainly trachea and lung (57, 81)

1.4.1.4. **MDV CXC chemokine (vIL-8)**

MDV genome encodes two spliced copies of viral CXC chemokine (136), named initially vIL-8 after sequence similarity to the first identified cIL-8 homolog (60, 98). The gene comprises of three exons and two introns; exon I encodes the first 22 N-terminal amino acids, represent a signal peptide with a cleavage site required for vIL-8 secretion. The secreted putative receptor binding portion of vIL-8 is encoded by exons II and III; contains the four positionally conserved cysteines residues in all CXC chemokines. With an aspartic acid–lysine–arginine

(DKR) motif before the first two conserved cysteines; vIL-8 classified within ELR- homeostatic CXC chemokine, whose members do not attract neutrophils and has an angiostatic effect (98).

vIL-8 as a secreted CXC chemokine recruits chicken peripheral blood mononuclear cells but not for heterophils (98), particularly vIL-8 act as a chemoattractant for B cells for lytic replication, as well as CD4+ CD25+ T cells as targets for MDV transformation and latent infection (43). However, the candidate receptor(s) that mediate vIL-8 recruitment of these cells has not been identified yet. Recently, it has been shown that the abrogation of vIL-8 expression without interfering with splicing with others neighboring genes and ORFs reduces MDV transformation (43), this may related to a decreased early cytolytic phase replication of MDV due to the absence of vIL-8.

Progenitor B cell as well as T cell express CXCR4; CXCL12/CXCR4 axis involved for homing of B and T cells to bursa and lymphoid organs during embryonic development (106). Mature B cells and follicular T cells express CXCR5, and CXCL13/CXCR5 required for homing of functional lymphocytes to the lymphoid organs and the site of infection (28). MDV may take advantage of these receptors in recruitments of target cells via vIL-8 secretion.

Another proposed functions of secreted vIL-8 are to antagonize chicken chemokines functions by blocking their receptors, or vIL-8 can binds to chemokine receptors and induce transcriptional and translational cascades that lead to migration of infected cells and enhance viral replication and pathogenicity (138).

1.4.2. **Chemokine receptors**

Chemokines mediate their biological functions via interaction with G-protein-coupled receptors, these receptors have characteristic seven alpha-helix transmembrane domains, are between 335-382 amino acids long, and have up to 81% amino acid identity **(Figure 7)** (85, 141).The nomenclature system of chicken chemokine receptors is designated by chemokine family that binds. Therefore, they classified to XCR, CCR, CXCR and CX3CR (85).

Chicken chemokine receptors are clustered in few chromosomal regions have their amino acid sequences encoded by a single exon (141). Chemokine receptors are distributed on various cells; some expressed exclusively on a small number of leukocyte types, and other are more broadly expressed (61).

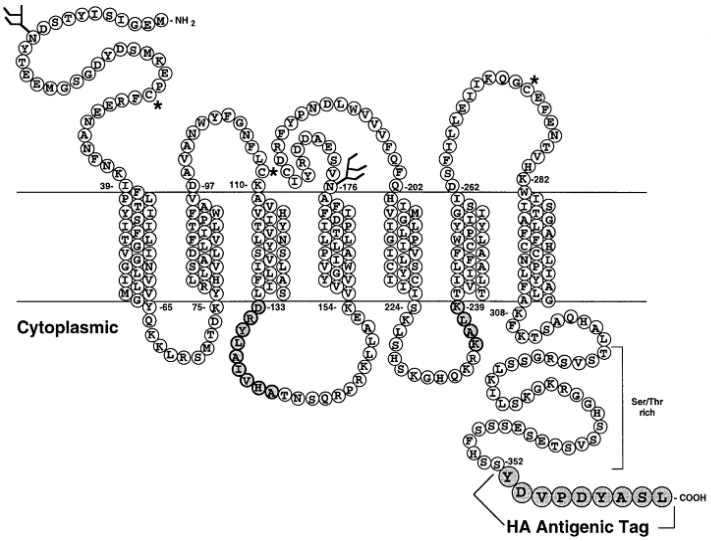

Figure 7: Diagrammatic representation of the CXC chemokine receptor

The structure of human CXCR4 involves the extracellular N-terminal acidic residues, cysteine residues are indicated by an asterisk. Glycosylation sites are shown. As other CXC chemokine receptors, the cysteine residues in extracellular loops 1 and 2 form a disulfide bond. C-terminal potential phosphorylation residues (serine and threonine) are shown. Obtained from Berson et al. (21)

Chicken have four CXC receptors: CXCR1, CXCR2, CXCR4, and CXCR5. CXCR1 is highly expressed on heterophils, monocytes, and macrophages but not in thymocytes (102), it appears to be functionally significant with ELR+ CXC chemokines particularly cCXCL8L1 and cCXCL8L2. Expression of cCXCR1 in mouse fibroblast NIH3t3 cell line, which naturally lack this receptor can activate calcium influx when cells treated with human CXCL8 or chicken cCXCL8L1 and cCXCL8L2 (72). It is important to note that cCXCR1 is not expressed in the thymus, liver, kidney, pancreas, heart, and lungs, which are the principal target organs for MDV life cycle and pathogenesis (102). cCXCR2 is also the target receptor for cCXCL8L2and related to the function of cCXCR1 (141). Chicken CXCR4 is expressed in bursa of Fabricius, as well as in the brain, liver, small and large intestines, chicken embryonic fibroblasts, and chicken blood leukocytes (73), this broad expression is consistent the role of CXCR4 in hematopoiesis, embryonic organ development of these organs (29). CXCL12(SDF-1) is the specific ligand, and CXCL12/CXCR4 pathway is involved in activation of B cell development in bone marrow and migration of B cell to bursa of Fabricius (106). In human, naïve human T cells express CXCR4, and it has a role in T cell differentiation (111). Endogenous expression of the chemokine CXCL12 (SDF-1) has an anti-apoptotic effect and stimulate the survival pathway based on its receptor CXCR-4 on DT40 chicken B cell (94). CXCR4 is also involved in viral infection and inflammation; it is the co-receptor for Human immunodeficiency virus (HIV) entry to CD4+ and overexpression of CXCR4 by the anti-

inflammatory effect of IL4 renders target cells for HIV infection (127). In contrast, stimulation of chicken T cells isolated from lymphoid organs and in splenocytes with concanavalin A results in CXCR4 down-regulation (28).

CXCR5 is the exclusive receptor of CXCL13, expressed on mature B cells and follicular helper T cells (28). In the mouse model, CXCL13/CXCR5 is required for initiation of lymph node development (137) and has been associated with recruitment of B and T cells and in the formation of ectopic lymphoid follicles in chronic infectious diseases (143). In chicken, CXCL13/CXCR5 is required for homing of B and follicular helper T cells to secondary lymphoid organs. High level of CXCR5 mRNA was detected in CD4+ cells isolated from bursa and thymus (28). Upon stimulation with Lipopolysaccharides (LPS) or Concanavalin A (Con A), CXCR5 mRNA is up-regulated in chicken CD4+ cells, it is also up-regulated in response to avian influenza virus infection, support the role in inflammatory and immune defense through B and T-cell migration (120).

1.5. Project introduction

1.5.1. Evolutionary and functional characterization of vIL-8

Marek's disease virus (MDV) has been shown to harbor genes that have a high sequence similarity to host genes. During its long co-evolution with the chicken, the virus likely 'pirated' these genes from the host and adapted them for the benefit of the virus. MDV genome encodes a viral CXC chemokine (virokine) named initially viral interleukin 8 (vIL-8) after sequence similarity to the first identified chicken CXC chemokine (cIL-8). The differences between vIL-8 and cIL-8 at structure and protein similarity as well as the type of recruited cells require identifying the true orthologue. Several other chicken CXC chemokines have been identified after the release of the complete chicken genome. CXCL13 has higher homology to vIL-8 and candidate to be the true orthologue. We aim to determine the actual orthologue and functional characterization of vIL-8.

The two main goals were:

1. Identification of evolution of vIL-8 through the phylogenic tree, gene and amino acid analysis with relation to chicken CXC chemokines.
2. Identification of the interaction between vIL-8 with respective chicken CXC receptors.

This knowledge is critical for understanding the role of virokine as well as host chemokine in MDV pathogenesis and for the host immune system.

1.5.2. **Role of vIL-8 in MDV replication and tumor formation**

To investigate if chicken CXCL13 can compensate a vIL-8 knockout in MDV replication and pathogenesis; a reverse genetic system of the highly virulent strain of MDV (RB1B) cloned in bacterial artificial chromosomes (BACs) was used. The three variants of chicken CXCL13 (CXCL13L1, CXCL13L2 or CXCL13L3) genes were inserted in RB1B that does not express vIL-8. Recombinant viruses expressing the secrete chicken CXCL13 instead of vIL8 were evaluated *in vitro* and *in vivo*.

The two main goals were:

1) Generation of recombinant MDV that replicate comparable to wild type virus and secrete chicken CXCL13.
2) Investigation if chicken CXCL13 can compensate vIL-8 role in MDV replication and pathogenesis.

2. Materials and Methods

2.1. Materials

2.1.1. Established cell lines, bacterial strains and insect cell

Name	Feature	References
Bacteria		
Bacterial strains were grown at 32 or 37°C in Luria-Bertani (LB) broth with shaking (220 rpm), and statically on LB agar plates with appropriate antibiotics.		
GS1783	DH10B λcI857 Δ(cro-bioA)<>araC-P BAD, I-SceI	Tischer et al.(135)
TOP10	F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(StrR) endA1 λ-	Invitrogen
DH10	F-endA1 deoR+ recA1 galE15 galK16 nupG rpsL Δ(lac) X74φ80lac ZΔM15ara D139Δ(ara,leu)7697mcrA Δ(mrr-hsd RMS-mcrBC) StrR λ–	Invitrogen
Insect cells		
SF9 cells were grown at 27°C in Insectomed SF express (Biochrome) with shaking (200 rpm).		
Sf9	Spodoptera frugiperda	Invitrogen
Cell lines		
CECs	Chicken embryo fibroblasts cells, VALO Specific pathogen-free (SPF) strain	Primary cells
DT40	Avian leukosis virus (ALV) induced bursal lymphoma cell line derived from a Hyline SC chicken.	Baba et al. (14)
HEK293	Human embryonic kidney cell line	ATCC® CRL-1573™

2.1.2. Chemicals and reagents

Product	Supplier
1Kb plus Ladder	Generuler TM, Thermo Scientific
Acetic acid(CH3COOH)	VWR, Dresden
Acetone	Applichem, Darmstadt
Acrylamid	Carl-Roth, Karlsruhe

Agar Agar(pure)	Carl-Roth, Karlsruhe
Agarose- Standard Roti® grade	Carl-Roth, Karlsruhe
Ammoniumperoxodisulfat (APS)	Carl-Roth, Karlsruhe
Ampicillin Sodium-salt	Carl-Roth, Karlsruhe
Arabinose L (+)	Carl-Roth, Karlsruhe
Bromophenol blue (C19H10Br4OS) 5	Alfa Aesar, Thermo Fisher Scientific.
Albumin bovine fraction V (BSA)	Applichem, Darmstadt
Chicken serum	Sigma-Aldrich, St. Louis
Chloramphenicol	Carl-Roth, Karlsruhe
Coomassie Brilliant Blue G solution	Sigma-Aldrich, St. Louis
Diethylpyrocarbonate (DEPC)	Applichem, Darmstadt
Dimethyl sulfoxide (DMSO)	Merck, Darmstadt
di-Sodium Hydrogenophsohate dodecahydrate	Applichem, Darmstadt
Dulbecco's MEM (DMEM)	Biochrom AG, Berlin
EDTA (ethylendiamine tetraacetic acid)	Applichem, Darmstadt
Ethanol. Absolute	Applichem, Darmstadt
Ethidium bromide 1%	Carl-Roth, Karlsruhe
FACS Clean	BD, San Jose
FACS Rinse	BD, San Jose
Fetal Bovine Serum (FBS)	Biochrom AG, Berlin
Formamide deionized Molecular biology grade	Applichem, Darmstadt
Fugene HD	Promega, Mannheim
Glycerol	Applichem, Darmstadt
HisPur™ Ni-NTA Resin	Thermo Scientific
Hydrochloric acid (37% HCl)	Carl-Roth, Karlsruhe
Hygromycin B Gold	Invivogen
Imidazole (C3H4N) 2	Serva, Heidelberg
Insectomed SF express.	Biochrome
Iscove`s basal medium	Biochrom AG, Berlin
Isopropyl alcohol (2-propanol)	Applichem, Darmstadt
Kanamycin sulphate	Carl-Roth, Karlsruhe
Magnesium chloride (MgCl2)	Merck, Darmstadt
Methanol	VWR, Dresden
Minimum essential Medium Eagle (MEM)	Biochrom AG, Berlin
Non-Essential Amino acids (NAE)-100x	Biochrom AG, Berlin
Nonfat dried milk powder	Applichem, Darmstadt
Opti-MEM I	Invitrogen
Paraformaldehyde	Sigma-Aldrich, St. Louis
Penicillin	Applichem, Darmstadt
Pepsine from porcine gastric mucosa	Sigma-Aldrich, St. Louis
peqGOLD dNTP-Mix	PeqLab, Erlangen
Phenol:Chloroform:Isoamylalcohol	Applichem, Darmstadt
Polyethylenimine, Linear, MW 25,000 (PEI)	Polysciences, Warrington, USA
Potassium acetate (KCH3CO2)	Applichem, Darmstadt
Potassium chloride (KCl)	Applichem, Darmstadt
Potassiumphosphate dibasic (K2HPO)	Applichem, Darmstadt
Potassiumphosphate monobasic (KH42PO) 4	Applichem, Darmstadt
Protein Prestained plus	Generuler TM, Thermo Scientific
RPMI 1640	Biochrom AG, Berlin
SDS (sodium dodecyl sulfate)	Sigma-Aldrich, St Louis

Sodium chloride (NaCl)	Applichem, Darmstadt
Sodium dihydrogenphosphate (NaH2PO)	Applichem, Darmstadt
Sodium deoxycholate detergent	Sigma-Aldrich, St Louis
Sodium hydroxide (NaOH)	Merck, Darmstadt
Sodium hydroxide pellets, extra pure 4)	Applichem, Darmstadt
Sodium Phosphate, monobasic, monohydrate	Sigma-Aldrich, St Louis
Sodium Pyruvate	Biochrom AG, Berlin
Sodiumhydrogencarbonate (NaHCO3)	Applichem, Darmstadt
Streptomycin	Applichem, Darmstadt
Tetracyclin	Applichem, Darmstadt
Tetramethylethylendiamin(TEMED)	Carl-Roth, Karlsruhe
Tris	Applichem, Darmstadt
Trichloroacetic acid	Carl-Roth, Karlsruhe
tri-Sodium Citrate dehydrate	Applichem, Darmstadt
Triton X-100	Merck, Darmstadt
Trypsin/EDTA 0,05%	GIBCO, Life Technologies, Grand Island
Tryptone	Carl-Roth, Karlsruhe
Tween-20	Carl-Roth, Karlsruhe
Water Molecular biology grade	Applichem, Darmstadt
Yeast Extract	Carl-Roth, Karlsruhe
β-mercaptoethanol ((HSCH2CHOH)2	Serva, Heidelberg

2.1.3. **Equipments and consumables**

Equipment	Manufacturer(Supplier)
ABI Prism 7500 Fast Real-time PCR system	Invitrogen
Analytical balance (Cubis MSA)	Sartorius AG , Germany
Bacterial Incubators Classic Line	Binder, Turtlingen
Bunsen burner (Article no. 1020)	Usbeck, Radevormwald
Centrifuges 5424/ 5424 R	Eppendorf AG, Hamburg
CO2 tissue culture incubator (New Brunswick™ Galaxy® 170 R)	Eppendorf AG, Hamburg
Electrophoresis Power Supply(Power Source ™ 300V)	VWR International, West Chester
FACS Aria II flow cytometers	BD Biosciences, USA.
BD FACSCanto™II	BD Biosciences, USA.
FIREBOY	INTEGRA Biosciences AG
Flexcycler Thermocycler	Analytik Jena, Jena
Freezer -20 ◦C (GN 3613)	LIEBHERR, Germany
Freezer –80 ◦C (HERA Freeze™ HFU B Series)	Thermo Scientific
Fume cupboards (DELTAguard)	Wesemann, Germany.
Genepulser Xcell Electroporator	Bio-Rad, Munich
Gradient Thermocycler	Biometra GmbH, Germany.
Insect cell shaker (New Brunswick™ Excella® E24)	Eppendorf AG, Hamburg
Laminar Flow Safety cabinet ClassII	Bleymehl Reinraumtechnik, Germany

Liquid nitrogen tank	Air liquide, Düsseldorf
Fluorescence inverted microscope	Carl Zeiss MicroImaging GmbH, Jena
Microscope (Motic AE20)	Scientific Instrument Company, Inc.
Microwave oven (Clatronic700 W)	Re-In. GmbH, Germany
Mr. Frosty™ Freezing Container	Thermo Scientific
New Brunswick™ Innova® 44(shaker bacterial incubator)	Eppendorf AG, Hamburg
Orbital shaker (OS-10)	PeqLab, Erlangen
pH–meter(inoLab pH Level 1)	Wissenschaftlich-Technische Werkstätten, Germany
Pipetboy	INTEGRA Biosciences AG
Pipettes (single–channel, multichannel)	VWR International, West Chester
Semi-Dry-Electro Blotter	PeqLab, Erlangen
Spectrophotometer (Smart spect Plus)	Bio-Rad, USA.
T-Gradient Thermocycle	Biometra, Göttingen
ThermoMixer® C	Eppendorf AG, Hamburg
UV Transiluminator	PeqLab, Erlangen
Vortex Genie 2™	Bender&Hobein AG, Zurich
Water bath	Eppendorf AG, Hamburg
Consumables	
0.2 ml thin-walled PCR tube	Applied Biosystems, UK
riplate® deepwell plates (1 ml)	Carl-Roth, Karlsruhe
Amicon Ultra-0.5 centrifugal filter unit (10-kDa cutoff),	Millipore- Merck KGaA, Darmstadt.
Cell culture dish 50, 100 and 150 mm	Sartsedt, Nümbrecht
Cell culture plates, 6-wel-flat base	Sartsedt, Nümbrecht
Cell–culture flasks (T–25, T–75)	Sartsedt, Nümbrecht
Cryotubes 1.8 ml	Sartsedt, Nümbrecht
Elektroporationküvette, 1 mm. steril	Biodeal, Markkleeberg
Eppendorf tubes (1.5, 2ml)	Sartsedt, Nümbrecht
VISKING® Dialysis Tubing (MWCO 12000 - 14000).	Serva, Heidelberg
Falcon bacteria (13ml)	Sartsedt, Nümbrecht
Falcon tubes (15 ml, 50 ml)	BD Falcon, Heidelberg, GER
Parafilm® M	Bems, Neenah
PVDF membrane	Carl-Roth, Karlsruhe
Whatman blotting paper	GE Healthcare, Freiburg
BD Falcon 12 x 75 mm Tube with Cell Strainer Cap	BD Biosciences, USA.
Petri dish 35x10mm with cams	Sartsedt, Nümbrecht
Pipette tips (1000, 200, 100 and 10)	VWR International, West Chester

Sterile Pipettes (5, 10, 25 ml)	Sartsedt, Nümbrecht
Transfection polypropylene tubes (round, short 17.1 x 105)	TPP, Trasadingen

2.1.4. **Media and buffers**

Bacterial media

Luria-Bertani (LB) Broth and agar Plates (Prepared centrally in our laboratory)	
LB Broth 1.0% Tryptone 0.5% Yeast Extract 1.0% Sodium Chloride	**SOB medium** 0.2% Tryptone 0.5% Yeast Extract 0.0584%g NaCl 0.0186%g KClH 7
SOC medium SOB medium 20 mM Glucose	**LB agar plates.** 15% Agar /LB Broth (w/v)
Mixture was autoclaved and cooled to approximately 60ºC before adding antibiotic and pouring aseptically into plates. Plates were stored at 4ºC in the dark.	

Tissue culture media

CECs(10% growth, 0.5% maintenance)	
Minimum essential Medium Eagle (MEM) Penicillin/Streptomycin (1000U/ml) FBS	500 ml 5 ml (1%) 50 ml (growth)/ 2.5 ml maintenance)
DT40	
Iscove`s basal medium Penicillin/Streptomycin (1000U/ml) Chicken serum	500 ml 5 ml (1%) 1ml
HEK293	
DEME Penicillin/Streptomycin (1000U/ml) FBS	500 ml 5ml (1%) 50ml

Buffers

General buffers	
1x Phosphate saline buffer (1xPBS) 2 mM KH_2PO 10 mM Na_4HPO 137 mM NaCl 2,7 mM KLC, pH 7.3	**1x Tris-acetate-EDTA buffer (TAE)**(Diluted from 50X Stock) 40 mM Tris 1 mM $Na_2EDTAx2H2O$ 20 mM Acetic acid 99%, pH 8,0
0.8-1% Agarose Gel 80-100 mM Agarose 1x TAE buffer 4 µl Ethidium bromide	**6x DNA loading buffer** 0.2% Bromophenol blue 60% Glycerol 60 mM EDTA

2x HEPES buffered saline (2x HBS);
140 mM NaCl,
1,5 mM Na2EDTAx2HO
50 mM HEPES,
pH 7.05

Plasmid preparation solutions

Resuspension Buffer
(P1) pH 8.0
50 mM Tris HCL
10 mM EDTA
100 µg/ml RNAse

Lysis Buffer
(P2)
200 mM NaOH
1% SDS

Protein blots solutions.

RIPA buffer I (SDS-Page sample preparation)
RIPA I
50 mM Tris-HCl, pH 7.5
150 mM NaCl
1% Triton X-100
1% Na-desoxycholate
0.1 % SDS

RIPA II
RIPAI
1mM EDTA

10x SDS-Page running buffer
250 mM Tris
1.9 M Glycine
1% SDS

Western blot washing buffer
0.03% Tween20 PBS

2x Western blot stripping buffer
50 mM glycine
2% SDS
pH 2

6x SDS sample Loading buffer
0.35 M Tris-HCl (pH6.8)
10% SDS
30% glycerol
10% β-mercaptoethanol
0.6% bromophenol blue

Comassie stain
0.1% Coomassie Brilliant Blue
50% Methanol (v/v)
10% Glacial acetic acid (v/v)

Destain solution
50% Methanol (v/v)
10% Glacial acetic acid (v/v)

Protein Purification buffers.

Equilibration Buffer:
20mM sodium phosphate
300mM sodium chloride (PBS) with 10mM imidazole
pH 7.4

Wash Buffer:
PBS with 25mM imidazole
pH 7.4

Elution Buffer:
PBS with 250mM imidazole (pH 7.4)

2.1.5. **Kits**

Name	Cat.No.	Manufacture
GF-1 AmbiClean PCR/Gel Purification kit	[Cat. No. GF-GC-200]	Vivantis, Malaysia
Qiagen Plasmid Midi Kit	[Cat. No. 12145]	Qiagen, Hilden
Pierce® BAC Protein Assay	[Cat. No. 23225]	Thermo Scientific
Hi Yield Gel/PCR DNA kit	[Cat No. 30 HYDF100-1]	SLG, Gauting
ECL Prime Western Blotting Detection Reagent	[Cat No RPN2236]	Amersham Biosciences
E-Z96 96-well blood DNA isolation	[Cat No D1192-01]	Omega Biotek, USA

2.1.6. **Enzymes**

All enzymes used in this project were purchased from New England Biolabs, UK unless stated below or in the text.

Enzyme	
Antarctic Phosphatase (AP)	LongAmp® Taq DNA polymerase
*Bam*HI	*Hind*III
*BstB*I	RNase
*Cla*I	*Sma*I
*Cop*I	*Sal*I
*Dpn*I	T4 ligase
*EcoR*I	Taq DNA-Polymerase (PeqLab, Erlangen)
Fast AP (Alkaline Phosphatase)	*Xho*I
*Kpn*I	

2.1.7. **Plasmids, bacmids and BACs**

Plasmid	Features	Reference
pEP Kan-S	Mammalian expression vector; T7prom, f1 ori, SV40 ori, SV40 pr ,KanR, I-Sce-I restricion site, AmpR, ColE1 ori, NeoR	Tischer et al(135)
pVitro-2-Hygro-MCS	Mammalian dual expression vector; Catalog # pvitro2-mcs	InvivoGen
pcDNA3.1	Mammalian expression vector; Catalog #V790-20	Invitrogen
pCR3.1	Mammalian expression vector; Catalog # pCR®3.1	Invitrogen
CXCL13L1-pcDNA67v5-HisA (K139)	Chicken CXCL13L1 in mammalian expression vector; T7prom, f1 ori, pBR322 ori, AmpR,pCMV, pSV40, NeoR	Pete Kaiser
CXCL13L2-pcDNA67v5-HisA (K140)	Chicken CXCL13L2 in mammalian expression vector; T7prom, f1 ori,	Pete Kaiser

	pBR322 ori, AmpR,pCMV, pSV40, NeoR	
CXCL13L3-pcDNA67v5-HisA (K141)	Chicken CXCL13L3 in mammalian expression vector; T7prom, f1 ori, pBR322 ori, AmpR,pCMV, pSV40, NeoR	Pete Kaiser
CMV-CXCL13L1-Kana-pcDNA	Kanamycin resistance gene (*aphAI*) insertion in CXCL13L1pcDNA; transfer construct for CXCL13L1 insertion during two Red recombination mutagenesis.	This work
CMV-CXCL13L2-Kana-pcDNA	Kanamycin resistance gene (*aphAI*) insertion in CXCL13L2; transfer construct for CXCL13L2 insertion during two Red recombination mutagenesis.	This work
CMV-CXCL13L3-Kana-pcDNA	Kanamycin resistance gene (*aphAI*) insertion in CXCL13L3; transfer construct for CXCL13L3 insertion during two Red recombination mutagenesis.	This work
FC-His -pFastBac11	Transfer plasmids for baculovirus expressing 6xHis-Tag at the C terminus of human IgG FC (FC-His) as control protein.	Engel et al. (43)
CXCL13L1-FC-His -pFastBac11	Cloning of CXCL13L1 at CopI site of pFastBac11-CpoI-Fc-His, as transfer plasmids for baculovirus expressing of CXCL13L1-FC-6xHis.	This work
CXCL13L2-FC-His -pFastBac11	Cloning of CXCL13L2 at CopI site of pFastBac11-CpoI-Fc-His, as transfer plasmids for baculovirus expressing of CXCL13L2-FC-6xHis.	This work
CXCL13L3-FC-His -pFastBac11	Cloning of CXCL13L3 at CopI site of pFastBac11-CpoI-Fc-His, as transfer plasmids for baculovirus expressing of CXCL13L3-FC-6xHis.	This work
huFC-pCR3	Cloning of human Fc to be secreted by CXCL13L2 signal peptide at HindIII and SaI1 of huFC-pCR3	This work
CXCL13L1_huFC-pCR3	Cloning of CXCL13L1 at HindIII and SaI1 of huFC-pCR3	This work
CXCL13L2_huFC-pCR3	Cloning of CXCL13L1 at HindIII and SaI1 of huFC-pCR3	This work
CXCL13L3_huFC-pCR3	Cloning of CXCL13L2 at HindIII and SaI1 of huFC-pCR3	This work
vIL8_huFC-pCR3	Cloning of vIL8 at HindIII and SaI1 of huFC-pCR3	This work

Bacmid	Feature	Reference
DH10Bac	DH10 contains a baculovirus shuttle vector (bacmid)	Invitrogen
DH10Bac-CXCL13L1	Bacmid for baculovirus expression System of CXCL13L1-FC-6xHis.	This work
DH10Bac-CXCL13-L2	Bacmid for baculovirus expression System of CXCL13L1-FC-6xHis.	This work
DH10Bac-CXCL13-L3	Bacmid for baculovirus expression System of CXCL13L1-FC-6xHis.	This work
BAC	Feature	Reference
ΔIRL_RB1B	Deleted IRL in RB1B vv strain of MDV	Engel et al (43)
ΔMetvIL-8_ΔIRL_RB1B (ΔMet-vIL8)	ΔIR_RB1B with Methionie to Leucine in vIL8 start codon.	Engel et al(43)
CMV-CXCL13L1-UL45	Insertion of chicken CXCL13L1 at the C-terminus of UL45 of ΔMetvIL-8 under the effect of CMV promoter	This work
rev-CMV-CXCL13L2-UL45	Removal of chicken CMV-CXCL13L1 from the C-terminus of CMV-CXCL13L1-UL45	This work
CMV-CXCL13L2-UL45	Insertion of chicken CXCL13L2 at the C-terminus of UL45 of ΔMetvIL-8 under the effect of CMV promoter	This work
rev-CMV-CXCL13L2-UL45	Removal of chicken CMV-CXCL13L2 from the C-terminus of CMV-CXCL13L2-UL45	This work
CMV-CXCL13L3-UL45	Insertion of chicken CXCL13L3 at the C-terminus of UL45 of ΔMetvIL-8 under the effect of CMV promoter	This work
rev-CMV-CXCL13L3-UL45	Removal of chicken CMV-CXCL13L3 from the C-terminus of CMV-CXCL13L3-UL45	This work
CMV-CXCL13L1-UL35	Insertion of chicken CXCL13L1 at the C-terminus of UL35 of ΔMetvIL-8under the effect of CMV promoter	This work
rev-CMV-CXCL13L1-UL35	Removal of chicken CMV-CXCL13L1 from the C-terminus of CMV-CXCL13L-UL35	This work
CMV-CXCL13L2-UL35	Insertion of chicken CXCL13L2 at the C-terminus of UL35 of ΔMetvIL-8 under the effect of CMV promoter	This work
rev-CMV-CXCL13L2-UL35	Removal of chicken CMV-CXCL13L2 from the C-terminus of CMV-CXCL13L-UL35	This work
CMV-CXCL13L3-UL35	Insertion of chicken CXCL13L3 at the C-terminus of UL35 of ΔMetvIL-8 under the effect of CMV promoter	This work
rev-CMV-CXCL13L2-UL35	Removal of chicken CMV-CXCL13L2 from the C-terminus of CMV-CXCL13L-UL35	This work
CMV-CXCL13L1-Mini-F	Insertion of chicken CXCL13L1 as replacement of xgpt in Mini-F of	This work

	ΔMetvIL-8under the effect of CMV promoter	
CMV-CXCL13L2-Mini-F	Insertion of chicken CXCL13L2 as replacement of xgpt in Mini-F of ΔMetvIL-8under the effect of CMV promoter	This work
CMV-CXCL13L3-Mini-F	Insertion of chicken CXCL13L3 as replacement of xgpt in Mini-F of ΔMetvIL-8under the effect of CMV promoter	This work
TK-CXCL13L1-Mini-F	Insertion of chicken CXCL13L1 as replacement of xgpt under TK promoter in the Mini-F sequence of ΔMetvIL-8	This work
rev-TK-CXCL13L1-Mini-F	Removal of chicken CXCL13L1 from TK-CXCL13L1-Mini-F	This work
TK-CXCL13L2-Mini-F	Insertion of chicken CXCL13L2 as replacement of xgpt under TK promoter in the Mini-F sequence of ΔMetvIL-8	This work
rev-TK-CXCL13L2-Mini-F	Removal of chicken CXCL13L2 from TK-CXCL13L2-Mini-F	This work
TK-CXCL13L3-Mini-F	Insertion of chicken CXCL13L3 as replacement of xgpt under TK promoter in the Mini-F sequence of ΔMetvIL-8	This work
rev-TK-CXCL13L3-Mini-F	Removal of chicken CXCL13L3 from TK-CXCL13L3-Mini-F	This work
RB1B-vIL-8-His	Insertion of His tag at vIL-8 C terminus of ΔIRL_RB1B	This work
CXCL13L1-vIL8	Insertion of chicken CXCL13L1 at the C terminus of vIL-8 in ΔMetvIL	This work
revCXCL13L1-vIL8	Removal of chicken CXCL13L1 from CXCL13L1-vIL8	This work

2.1.8. **Primers and probes**

All primers and probes used in this work were designed using Vector NTI 9.1 software (Invitrogen), ordered and purchased from Integrated DNA Technologies (IDT, Coralville, USA).Sequencing reactions were performed by LGC Genomics GmbH (Berlin, Germany).

Primers	Sequence (5'-3')	
Cloning and sequencing of shuttle plasmids for two step red recombination.		
CXCL13s-Kana.	Forward (FW)	CCCAGGGGCCCTTCGAAGGTAAGCCTATCCCTAACCC TCTCCTAGGGATAACAGGGTAATCGATTT
	Reverse (RV)	CCTTCGAAGGGCCCGCCAGTGTTACAACCAATTAACC
Seq-CXCL13s-Kana	FW	GTTGATGCGCTGGCAGTGTTCCTG
	RV	CAGGAACACTGCCAGCGCATCAAC
Cloning of transfer plasmids for Baculovirus		
CXCL13L1_p	FW	GCGGCCGGTCCGCTGTCGCTGGAGAGCCTGC

FastBac11	RV	GGCTCCGGACCGGCTGGGACCGGTGGCTGTCTC
CXCL13L2_p FastBac11	FW	GCGGCCGGTCCGGCCATCCTGGAAGCCAACG
	RV	TGGGCTCCGGACCGGCCTGGGGAGCAGCTTTCTTCTTC
CXCL13L3_p FastBac11	FW	TGCGGCCGGTCCGGCCATCCTGGAAGTAAACGGAAAC
	RV	GGGCTCCGGACCGGCTCTTTTCTTCGTGCCAGCAATG
Cloning of transfer plasmids for HEK293		
huFC-pCR3	FW	GACCCAAAGCTTAGATGGCAGTGCGGGCAGCCCTGCTGCT GGGGCTGCTGCTGGTGGTTCTGTGCCCTGGGGGCGATGC GAAAACTCACACATGCCCACCG
	RV	CTGTGGTCGACTCATTTACCCGGAGACAGGGAGAGG
CXCL13L1_h uFC-pCR3	FW	ACCCAAAGCTTATGCGGGCGCTGCAGG
	RV	TGTCGAGTCGACCTGGGACCGGTGGCTGTCTC
CXCL13L2_h uFC-pCR3	FW	GACCCAAAGCTTAGATGGCAGTGCGGGCAGC
	FW	GTCGAGTCGACCCTGGGGAGCAGCTTTCTTC
CXCL13L3_h uFC-pCR3	RV	ACCCAAAGCTTATGCGAGTACTGGCGCGG
	FW	TGTCGAGTCGACCTCTTTTCTTCGTGCCAGCAATGC
vIL8_huFC-pCR3	FW	ACCCAAAGCTTATGCAGGCGTTGTTGCTAGTATTG
	RV	TGTCGAGTCGACCTAGACAGATATGGGAACCAATAGTAGG AG
Seq of pCR3	FW	GGTAGGCGTGTACGGTGGG
	FW	GGGGCAAACAACAGATGGC
Insertion of CXCL13L1 in vIL-8		
CXCL13L1-vIL-8	FW	GCAGGGGGTGTGGGTTTGATGAGCAGTTGGGGCGGCAAA AATGCGGGCGCTGCAGG
	RV	TATGAATAGAACCAATACTAGCAACAACGCCTGCAACTAAT GGTGATGGTGATGATGACC
revCXCL13L1-vIL-8	FW	GCAGGGGGTGTGGGTTTGATGAGCAGTTGGGGCGGCAAA AATGCAGGCGTTGTTGCTAGTGATAACAGGGTAATCGATTT A
	FW	TGTACTATGAATAGAACCAATACTAGCAACAACGCCTGCAT TTTTGCCGCCCCAACTGCTAGTGTTACAACCAATTAACC
Seq-CXCL13L1-vIL-8	RV	CTGCTATGCAGGGGTCGTGGGAA
	FW	GCACCTCTTGTCGACAGCGAGAC
Insertion of pCMV-CXCL13s in UL45		
CXCL13s-UL45	FW	AACGCAATTATGAAATAAACACAAAAATAAACACTATTCTTG GGCCAGATATACGCGTTG
	RV	GTGGGGCAGGACAGCAAAAAGCGGTTCTTTATTTCTTTAAT AATCGAGCATGAAAGGTG
revCXCL13s-UL45	FW	ACGCAATTATGAAATAAACACAAAAATAAACACTATTCTTAA AGCGGTTCTTTATTTCTTTAGGGATAACAGGGTAATCGATTT ATTC
	FW	CCTTTCATGCTCGATTATTAAAGAAATAAAGAACCGCTTTAA GAATAGTGTTTATTTTTGGCCAGTGTTACAACCAATTAACCA AT
Seq-CXCL13s-UL45	RV	GCGTTGTATGCCAACGCCC
	FW	CCTCGACTGTGCCTTCTA
Insertion of pCMV-CXCL13s in UL35		
CXCL13s-	RV	CATAATAAAGTACAACAATAAAGTGTTTAAACAGTATACCGG

UL35		GGCCAGATATACGCGTTG
	FW	GATCCTGACTGGATAAATAAAAAAGAATGGAATTAGGTGTTT GCTGTCCTGCCCCAC
revCXCL13s -UL35	FW	GATGATGATATATCGACGTCATAATAAAGTACAACAATAAAG TGTTTAAACAGTATACCGTAGGGATAACAGGGTAATCGATTT A
	RV	AGAATGGAATTAGGTGTCGGTATACTGTTTAAACACTTTATT GTTGTACTTTATTATGCCAGTGTTACAACCAATTAACC
Seq-CXCL13s-UL35	FW	GGCTTGAAACGCACATTCC
	FW	CATTGTATGCGCTTACAAAAGC
Insertion of CMV-CXCL13s in Mini-F		
pCMV-CXCL13s _Mini	FW	CGTATAGCATACATTATACGAAGTTATGGATCTGATGTACG GGCCAGATATACGCGTTG
	FW	AACGTCGACCCGGGTACCTCTAGATCCGCTAGCGCTTTAAT GGTGATGGTGATGATGACC
Seq-pCMV-CXCL13s _Mini	RV	CTGTCGAATAACAGCTAATGACTACC
	FW	TGGTCGAGAAGCTAGGCG
Insertion of TK-CXCL13s in Mini-F		
CMV-CXCL13L1-Mini-F	RV	GCATATTAAGGTGACACGCGCGGCCTCGAACACAGCTGCA GGCCATGCGGGCGCTGCAGGC
	FW	AACGTCGACCCGGGTACCTCTAGATCCGCTAGCGCTTTAAT GGTGATGGTGATGATGACC
CMV-CXCL13L2-Mini-F	FW	GCATATTAAGGTGACACGCGCGGCCTCGAACACAGCTGCA GGCCATGGCAGTGCGGGCAGC
	RV	AACGTCGACCCGGGTACCTCTAGATCCGCTAGCGCTTTAAT GGTGATGGTGATGATGACC
CMV-CXCL13L3-Mini-F	FW	CATATTAAGGTGACACGCGCGGCCTCGAACACAGCTGCAG GCCATGCGAGTACTGGCGCGG
	FW	AACGTCGACCCGGGTACCTCTAGATCCGCTAGCGCTTTAAT GGTGATGGTGATGATGACC
revCXCL13s-Mini-F	RV	CATATTAAGGTGACACGCGCGGCCTCGAACACAGCTGCAG GCCTAAAGCGCTAGCGGATAGGGATAACAGGGTAATCGAT TTA
	FW	CGTCGACCCGGGTACCTCTAGATCCGCTAGCGCTTTAGGC CTGCAGCTGTGTTCGAGGCCGCCAGTGTTACAACCAATTAA CC
Seq-CXCL13s-Mini-F	FW	GCCCAGCGTCTTGTCATT
	RV	TGGTCGAGAAGCTAGGCG
qPCR Primers		
ICP4	FW	CGTGTTTTCCGGCATGTG
	RV	TCCCATACCAATCCTCATCCA
iNOS	FW	GAGTGGTTTAAGGAGTTGGATCTGA
	FW	TTCCAGACCTCCCACCTCAA
qPCR Probes		
ICP4		FAM-CCCCCACCAGGTGCAGGCA-TAM
iNOS		FAM-CTCTGCCTGCTGTTGCCAACATGC-TAM

2.1.9. **Antibodies**

Antibody	Source	Concentration	Manufacture
Primary			
Anti MDV-vIL-8	Rabbit	1:5000	Cui et al. (36)
Anti 6xHis-tag	Rabbit	1:5000	Rockland, Limerik
Anti MDV US2, polyclonal	Chicken	1:5000	Jarosinski et al. (55)
Anti-glycoprotein C (anti-gC) antibody (clone 1A6)	Mouse	1:10000	Tischer et al (133)
Anti-chicken CXCR5	Mouse	1:100	Dr. Sonja Härtle
Secondary			
IF			
Anti-chicken IgG (H+L), Alexa Fluor® 488 conjugate	Goat	1:1000	Invitrogen
Anti-chicken IgG (H+L), Alexa Fluor®546 conjugate	Goat	1:1000	Invitrogen
FACS			
Anti-Mouse IgG (H +L), Alexa Fluor® 647 conjugate	Goat	1:1000	Invitrogen
Anti-Human IgG (H+L), Alexa Fluor® 647 conjugate	Goat	1:1000	Invitrogen
Western blot			
Anti-rabbit HRP	Goat	1:1000	Cell Signaling
Anti-mouse HRP	Goat	1:1000	Cell Signaling
Anti-human-HRP	Goat	1:2500	Invitrogen

2.1.10. **Software**

Name	Manufacture
EndNote.X5	THOMSON REUTERS.
Applied Biosystems 7500/7500 Fast Real-Time PCR System Software v(v2.0.6)	Invitrogen.
BLAST	NCBI, Bethesda, USA.
Chemi-Capt	Vilber-Lourmat, Eberhardzell.
Finch TV 1.4.0	Geospiza, Inc.
FlowJo 7.6.5,	FlowJo, LLC. Photos courtesy Graham Lewis.
DNA Copy Number and Dilution Calculator	Thermo Fisher Scientific, UK.
GraphPad Prism 5	GraphPad Software Inc, La Jolla
Image J 1.48 v	Schneider et al. (115)
MEGA6	Tamura et al. (129)
ND-1000 V.3.0.7	PeqLab, Erlangen
Vector NTI Advance™ 9.1	Invitrogen.

2.2. Methods

2.2.1. Standard Molecular biological methods

2.2.1.1. Bioinformatics analysis

Vector NTI Advance™ 9.1 (Invitrogen) was used for primers design, restrictions endonuclease mapping and sequence results alignment. Primers used for PCR amplification were designed according to the following criteria: (i) the GC content of a primer was close to 50 %, melting temperature (Tm) lies approximately between 50 and 58 °C, (ii) forward and reverse primers of each PCR reaction were designed to have approximately the same Tm, (iii) the length of primers for cloning and sequences were between 18 and 24 base pairs. (iv)The primers were designed to avoid self-complementation. Assembly of the nucleotide sequences and translation into amino acid sequences of the PCR products and recombinant mutants were also performed with Vector NTI Advance™ 9.1 software (Invitrogen).

2.2.1.2. Phylogenic trees and sequence analyses

Complete amino acid sequences of vIL-8 and currently known human and chicken CXC chemokines were retrieved from GenBank® (http://www.ncbi.nlm.nih.gov/genbank/), complete sequences of amino acid were imported to MEGA 6 (129) and Vector NTI Advance™ 9.1 (Invitrogen). The amino acid sequences were aligned using the ClustalW of MEGA 6 software (129) and Vector NTI Advance™ 9.1. Human and chicken CXC chemokines GenBank® accession number and protein ID are listed in **Table 3**. The phylogenetic tree was constructed from complete amino acid sequences, using the neighbor-joining method with Pairwise deletion and Poisson correction in MEGA 6 (129). The similarity of amino acid sequences was determined based on alignments with RB1B-vIL-8 as a reference profile.

Table 3: GenBank accession number and protein ID of human, chicken and MDV CXC chemokines

Full Name	Name	Species	GenBank	protein ID
chemokine (C-X-C motif) ligand 1-like [Gallus gallus (chicken)]	cCXCL1[1]	Gallus gallus	XM_420608.3	XP_420608.1
Gallus gallus interleukin 8-like 1 (IL8L1), mRNA	CXCL8L1[2]	Gallus gallus	NM_205018.1	NP_990349.1
IL8L2 interleukin 8-like 2 [Gallus gallus (chicken)]	CXCL8L2[3]	Gallus gallus	NM_205498.1	NP_990829.1

PREDICTED: Gallus gallus chemokine (CXCL13L2), transcript variant X4, mRNA	cCXCL13L1	Gallus gallus	XM_004941024.1	XP_004941081.1
Gallus gallus mRNA for chemokine (CXCL13L2 gene)	cCXCL13L2	Gallus gallus	FR874037.1	CCC15119.1
Gallus gallus mRNA for chemokine (CXCL13L3 gene)	cCXCL13L3	Gallus gallus	FR874038.1	CCC15120.1
Gallus gallus chemokine (C-X-C motif) ligand 12 (CXCL12), mRNA	cCXCL12	Gallus gallus	NM_204510.1	NP_989841.1
Gallus gallus chemokine (C-X-C motif) ligand 14 (CXCL14), mRNA	cCXCL14	Gallus gallus	NM_204712.2	NP_990043.1
vIL-8 protein [Gallid herpesvirus 2]	vIL-8	RB1B-GHV2	AF489269.1	AAN60433.1
Homo sapiens chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha), mRNA (cDNA clone MGC:9049 IMAGE:3856841), complete cds	hCXCL1	Homo sapiens	BC011976.1	AAH11976.1
Homo sapiens chemokine (C-X-C motif) ligand 2, mRNA (cDNA clone MGC:23071 IMAGE:4875789), complete cds	hCXCL2	Homo sapiens	BC015753.1	AAH15753.1
Homo sapiens chemokine (C-X-C motif) ligand 3, mRNA (cDNA clone MGC:72105 IMAGE:4717346), complete cds	hCXCL3	Homo sapiens	BC065743.1	AAH65743.1
platelet factor 4 precursor [Homo sapiens]	hCXCL4	Homo sapiens	NM_002619.3	NP_002610.1
Homo sapiens chemokine (C-X-C motif) ligand 5, mRNA (cDNA clone MGC:12304 IMAGE:3826939), complete cds	hCXCL5	Homo sapiens	BC008376.1	AAH08376.1
Homo sapiens chemokine (C-X-C motif) ligand 6 (granulocyte chemotactic protein 2), mRNA (cDNA clone MGC:21713 IMAGE:4471095), complete cds	hCXCL6	Homo sapiens	BC013744.1	AAH13744.1
Homo sapiens pro-platelet basic protein (chemokine (C-X-C motif) ligand 7), mRNA (cDNA clone MGC:40008 IMAGE:5214257), complete cds	hCXCL7	Homo sapiens	BC028217.1	AAH28217.1
Homo sapiens chemokine (C-X-C motif) ligand 8 (CXCL8), mRNA	hCXCL8	Homo sapiens	NM_000584.3	NP_000575.1

Homo sapiens chemokine (C-X-C motif) ligand 9, mRNA (cDNA clone MGC:71972 IMAGE:30370826), complete cds	hCXCL9	Homo sapiens	BC063122.1	AAH63122.1
Homo sapiens chemokine (C-X-C motif) ligand 10, mRNA (cDNA clone MGC:13622 IMAGE:4274617), complete cds	hCXCL10	Homo sapiens	BC010954.1	AAH10954.1
Homo sapiens chemokine (C-X-C motif) ligand 11, mRNA (cDNA clone MGC:13317 IMAGE:4096572), complete cds	hCXCL11	Homo sapiens	BC012532.1	AAH12532.1
Homo sapiens chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1), mRNA (cDNA clone MGC:47612 IMAGE:5729604), complete cds	hCXCL12	Homo sapiens	BC039893.1	AAH39893.1
Homo sapiens chemokine (C-X-C motif) ligand 13, mRNA (cDNA clone MGC:13521 IMAGE:4246053), complete cds	hCXCL13	Homo sapiens	BC012589.1	AAH12589.1
PREDICTED: Pan troglodytes chemokine (C-X-C motif) ligand 14 (CXCL14), mRNA	hCXCL14	Homo sapiens	XM_527018.5	XP_527018.2
no human CXCL15		Homo sapiens		
Homo sapiens chemokine (C-X-C motif) ligand 16, mRNA (cDNA clone MGC:26644 IMAGE:4837010), complete cds	hCXCL16	Homo sapiens	BC017588.1	AAH17588.1

2.2.1.3. **Polymerase Chain Reaction (PCR)**

PCR was carried out to amplify DNA sequences for cloning, sequencing and Two step Red-recombination mutagenesis. For routine PCR, reactions were set up using the supplied components and were made up to a final volume of 20 or 50 µl in a 0.2 ml thin-walled PCR tube in Flexcycler Thermocycler (Analytik Jena) as following:

Table 4: *Taq* DNA routine PCR reaction component

Component	20µl	50µl	Final concentration
10x Standard *Taq* Reaction Buffer	2.5 µl	5µl	1x
10 mM dNTPs	0.5 µl	1µl	200µM
Forward primer (100 pmol/µl)	0.5 µl	1µl	0.2 µM
Reverse primer (100 pmol/µl)	0.5 µl	1µl	0.2 µM
DNA template			<1ng
Taq DNA Polymerase	0.125 µ	0.25	1.25 units/50 µl PCR
dH2O	to 20 µl	to 50 µl	

One-step routine PCR was used for short overhang product and for colony PCR screening. *Taq* DNA Polymerase (PeqLab, Erlangen) was used .in a Flexcycler Thermocycler (Analytik Jena) according to the following thermocycling conditions:

Table 5: Thermocycling conditions for a routine PCR

PCR Step		Temperature (°C)	Time
1	Initial denaturation	94 °C	5 min
2	Denaturation	94 °C	30 s
3	Annealing	52-58°C	45 s
4	Extension	72 °C	1 min for 1 Kbp
Cycle from step 5 to step 4 30x			
10	Holding	4°C	

Mutagenesis PCR involves the synthesis of homologues site for recombination, such a long overhang (>20bp) was performed by high fidelity amplification of DNA using LongAmp® Taq DNA polymerase (New England Biolabs, UK).

Table 6: LongAmp PCR reaction component

Component	50µl	Final concentration
5X LongAmp *Taq* Reaction Buffer	10µl	1x
10 mM dNTPs	1.5µl	300µM
Forward primer (100 pmol/µl)	2µl	0.4 µM
Reverse primer (100 pmol/µl)	2µl	0.4 µM
DNA template		<1ng
LongAmp *Taq* DNA Polymerase	2µl	5 units/50 µl PCR
dH2O	to 50 µl	

All reactions were performed by 2-step PCR and carried out in a Flexcycler Thermocycler (Analytik Jena) according to the following thermocycling conditions:

Table 7: Thermocycling conditions for a LongAmp DNA polymerase 2-step PCR

PCR Step		Temperature (°C)	Time
1	Initial denaturation	94 °C	5 min
2	Denaturation	94 °C	30 s
3	Annealing	52-58°C	45 s
4	Extension	72 °C	1 min for 1 Kbp
Cycle from step 2 to step 4 10x			
5	Denaturation	94 °C	30 s
6	Annealing	68 °C	45 s
7	Extension	72 °C	1 min for 1 Kbp
8	Final extension	72 °C	30 s
Cycle from step 5 to step 4 30x			
10	Holding	4°C	

2.2.1.4. DNA isolation and purification

Contaminant primer, nucleotides, polymerase or salts were removed from PCR reactions using the GF-1 DNA extraction kit (Vivantis, Malaysia) according to manufacturer instructions.

2.2.1.5. Agarose gel electrophoresis

To visualize the PCR products or fragments of DNA for restriction fragment length polymorphisms (RFLP), samples were run on agarose gel. Agarose gel was prepared by mixing either 0.8 or 1 g of Agarose per 100ml of TAE buffer (0.8 to 1 % (w/v)) and heating in a microwave till completely been dissolved. The mixture was then allowed to cool slightly and 5µl of ethidium bromide stock solution (10 mg/ml) was added and mixed thoroughly. The mixture was then poured into a block mould with combs for placement of the nucleic acid samples. Once cooled, the combs were removed and the gel block was placed in an electrophoresis tank filled with 1X TAE buffer with the sample wells situated at the negative pole of the tank. Nucleic acid samples were loaded into the wells combined with 6 X DNA loading buffer. The current was applied at the rate of 100-120V for 30-60 minutes. After running, gels were visualized using UV Transilluminator (PeqLab, Erlangen) and photographed. 1 kb-plus DNA ladder (Invitrogen) was used to determine the DNA fragment size on all agarose gels.

2.2.1.6. Restriction endonuclease

For analysis of plasmids and recombinant BAC clones, restriction enzyme digestion was carried out using a broad range of restriction endonuclease. DNA was digested according to the manufacturer's instructions, using the recommended buffers and conditions.

2.2.1.7. DNA gel extraction

Purification of DNA fragments from agarose gels was performed by identifying the band of interest from the gel using long wavelength UV light. The band was then carefully cut from the agarose gel with a surgical scalpel and DNA purified using the GF1 DNA extraction kits according to standard manufacturer's instructions (Vivantis, Malaysia).

2.2.1.8. Determination of DNA-concentration by spectrophotometry

Concentration and purity of the purified DNA sample were determined using Nanodrop ND-1000 low volume spectrophotometer measuring 220nm to 750nm wavelength and version 3.7.1 software (PeqLab, Erlangen). The 260/280 absorbance was used as an indicator for contaminants of the DNA sample.

2.2.1.9. Dephosphorylation

Antarctic Phosphatase (AP) (New England Biolabs, UK) was used to prevent self-ligation by removing of 5' phosphate groups. In brief, digested plasmid DNA with a single enzyme was incubated with AP in the recommended buffer for 1 hour at 37 °C. The AP was inactivated by incubation at 65^{o}C for 15 min.

2.2.1.10. DNA ligation

All DNA Ligation were done by T4 DNA Ligase (New England Biolabs, UK) according to manufacturer instruction. Ligation reaction compromises a molar ratio of 1:3 vector to insert was calculated by NEBioCalculator (http://nebiocalculator.neb.com). A 1-5 µl of the ligation reaction was used to transform *E.coli* competent cells (Section 2.2.2.3).

2.2.1.11. Colony PCR

A single bacterial colony was used directly as template for PCR to detect correct clones after ligation reaction. Single colony was transferred to a new agar plate with appropriate antibiotic by a pipette tip and the remains of the cells in the tip were mixed into the PCR reaction. The *Taq* DNA Polymerase (PeqLab, Erlangen) was set up as described earlier with a total volume adjusted to 20µl without DNA template.

2.2.1.12. **DNA sequencing**

For plasmid DNA sequencing and the PCR amplicons covering the target sequence in MDV genome were sent according to the following template DNA amount:

Plasmids		1000 ng
PCR product	200 - 500 bp	100 ng
	500 - 1,000 bp	200 ng
	1,000 - 2,000 bp	400 ng

10 µl DNA mixed with 2 µl of primer cover the target sequence in a total of volume of 14µl was sent to LGC Genomics GmbH (Berlin, Germany). Sequencing results were analyzed using Finch TV chromatogram to view DNA sequence traces (Geospiza, Inc.) and Vector NTI version 9.1.0software (Invitrogen) to combine, align and overlay individual sequences.

2.2.2. Microbiological Methods

2.2.2.1. Bacterial culture

Laboratory strains of *E. coli* were grown in Luria-Bertani (LB) medium (Section 2.1.1) or plated on LB agar plates. The LB media and agar were sterilized by autoclaving. LB agar was supplemented with the appropriate antibiotics and poured into 10 cm diameter Petri dishes. Different antibiotics were used in this project, for example: Hygromycin (hyro) (200µg/ml), ampicillin (amp) (100 µg/ml), kanamycin (kan) (50 µg/ml), chloramphenicol (cam) (20µg/ml) and gentamicin (gent) (7 µg/ml). Bacteria were plated on LB agar by streaking with a wire loop or spreading with glass beads and incubated overnight inverted at 32^{o}C or 37^{o}C. Single colonies were inoculated in sterile liquid LB medium supplemented with the appropriate antibiotic. Liquid cultures were incubated at 32^{o}C or 37^{o}C overnight in the bacterial incubator with shaking at 220 rpm.

2.2.2.2. Preparation of chemically competent *E. coli* (TOP10 and DH10B)

For the preparation of chemically competent *E. coli* eitherTOP10 or DH10B cells, a single colony was inoculated in 5 ml LB medium and incubated overnight at 37 °C with shaking at 220 rpm. Then, 1 ml of the overnight culture was inoculated in 100 ml LB in a 500 ml Erlenmeyer flask and incubated at 37°C with shaking at 220 rpm till the logarithmic growth of the bacteria reached to 0.45-0.6 $O.D_{600}$. The culture was transferred to pre-chilled 50 ml conical bottom centrifuge tubes and incubated for 5-10 min on ice. The bacteria were then pelleted at 4°C for 2 min at 1000g and resuspended in 10 ml ice-cold 50 mM CaCl2. After the centrifugation, the supernatant was discarded and the bacterial pellet was resuspended in 10 ml ice-cold 50 mM CaCl2 and incubated on ice for 30 min. The resuspended cells were centrifugated at 400g for 5 min at 4°C and then the bacterial pellet was resuspended in 2 ml ice-cold 100 mM CaCl2-15% glycerol. Competent bacterial strains were either immediately used or stored at -80°C for later use.

2.2.2.3. Chemical transformation of chemically competent *E. coli*

For transformation of the competent *E.coli,* 0.5-1 µg of plasmid or ligation mixture (usually 1-5µl) was added to *E.coli.* and then incubated on ice for 30 minutes. Heat shock was performed in a water bath at 42°C for 90 Sec, followed by 2 min incubation on ice. Bacteria were grown in 1 ml SOC medium for 1 h at 37°C and then plated on LB agar plate with the appropriate antibiotics overnight at 37°C. Individual colonies were replica-plated onto a master plate and inoculated in LB broth culture for screening purposes. Colony PCR and DNA restriction endonuclease digestion with appropriate enzymes were used to check the

correct clones. Clones that were positive by colony PCR, and presented the expected restriction digest pattern, were sequenced with appropriate primers.

2.2.2.4. Preparation of electrocompetent *E.coil*

The *E. coli* strain GS 1783 containing the MDV- Bacterial Artificial Chromosome (BAC) was grown overnight at 32°C in 5 ml of LB-cam. A 2ml of the overnight culture were inoculated in 100 ml of fresh LB-cam and then incubated at 32°C till the OD_{600} reached to 0.5-0.6. The bacterial cultures were transferred to a 42°C water bath for 15 min with shaking at 220 rpm in order to induce the Red recombination system. After heating, cultures were transferred to a water-ice bath and incubated for 20 min with shaking at 220 rpm. Bacteria were pelleted by centrifugation for 2 min at 5000 rpm at 4°C. Pellets were washed three times with ice-cold 10% glycerol in sterile millipore water and resuspended in 50 µl of 10% glycerol. A100 ng of purified DNA product was used for electroporation. Electroporation was carried out at 1.6 kV, 25 µF and 200 Ω. Samples were resuspended in 1000 µl of pre-warmed SOC medium and incubated for 3 h at 32°C with shaking at 220 rpm. The bacterial pellet was resuspended in 100 µl of the SOC and plated on selective LB agar plates.

2.2.2.5. Small-scale plasmid DNA isolation from bacteria (Mini-prep)

A single *E. coli* colony was inoculated in 5 ml of LB medium with the appropriate antibiotic and incubated overnight at 32°C in the bacterial incubator with shaking at 220 rpm. The bacterial pellet was resuspended in 300 µl of solution P1. The bacterial cells were lysed by incubation in 300 µl of the P2 solution at room temperature (RT) for 5 min and then neutralized with 300µl of solution P3. The precipitated solution was incubated on ice for 10 min. and centrifuged at 13000xg at 4°C for 10 min. The supernatant was transferred into a new tube, mixed with 800µl Phenol:Chloroform:Isoamylalcohol on the vortex and then centrifuged at 13000xg for 10 min. The upper phase (~700µl) was transferred to fresh tubes and a 700 µl isopropanol was added. The DNA pellet was precipitated by centrifugation at maximum speed for 15 min at 4°C. The DNA pellet was washed twice with 800 µl 70% ethanol and dissolved in 30 µl of TE-RNase buffer.

2.2.2.6. Medium-Scale plasmid DNA isolation from bacteria (Midi-prep)

Medium-scale isolation of plasmid or BAC DNA was carried out using QIAGEN Plasmid Midi Kit (Qiagen, UK) according to the manufacturer's instructions. Briefly, a single colony was isolated and inoculated into 3 ml LB medium supplemented with the appropriate selective antibiotic. The bacterial culture was incubated at 32°C with shaking at 220 rpm. After 8 hours incubation, 1 ml of the bacterial culture was used to inoculate 200 ml fresh LB medium supplemented with the appropriate antibiotic in sterile 1000 ml Erlenmeyer flask. The culture

was incubated overnight at 32°C with shaking at 220rpm. Bacterial culture was then pelleted by centrifugation at 5000 x g for 15 minutes at 4 °C. The bacterial pellet was resuspended in 10 ml P1 with RNase and lysed by mixing and vigorous inversion with 10 ml of r P2 for 5 min at RT. The lysis reaction was neutralized by addition of 10 ml P3, followed by vigorous inversion and incubation for 30 min in ice. The lysate was poured using QIAGEN-tip column after equilibration with 5 ml of equilibration buffer, column allows emptying by gravity and washed two times with 10 ml washing buffer. DNA was eluted in 5 ml of 65 °C preheated elution buffer and participated with 3.5 ml isopropanol. DNA pellet was washed with 70% ethanol two times and dissolved in 30 µl of TE-RNase. DNA concentration was measured by Nanodrop 1000 (PeqLab, Erlangen).

2.2.2.7. **MDV Bacterial Artificial Chromosome (BAC)**

Bacterial Artificial Chromosome is based on cloning of self-replication machinery factor of bacteria (F plasmid) in a DNA constructs; such as full-length viral genome, and transform it into other bacteria mainly E coli.. Several herpesvirus genomes have been cloned in BAC including different strains of MDV (101, 107, 116). It has been previously reported that insertion of the Mini-F sequence in a non-essential location of MDV genome US2 did not affect viral replication *in vitro* and *in vivo* (35, 88). BAC technology provides a valuable advance tool for stable maintenance of MDV genome in *E.coli*. In addition, it allows efficient manipulation of viral genomes by mutagenesis. The virus can be reconstituted by transfection of BAC DNA into in susceptible cells. It is also allow to visualization of tagged BAC-encoding viral sequences in transfected cells (132). Generally, this tool has an essential role in the study of viral pathogenesis and efficiently facilities vaccine development studies. RB1B-MDV BAC was used in this study to insert CXCL13s in order to be expressed as secreted chemokine to mimic vIL-8 secretion, as well as to generate the revertant mutants by removing the inserted sequence to ensure the stability of BAC during mutagenesis steps. The mutants were reconstituted to be characterized *in vitro* and *in vivo*. Recombinant MDV BAC harboring the chicken CXCL13s was constructed using pRB1B and two-step en-passant Red recombination previously developed in our lab (134, 135).

For insertion of chicken CXCL13s, in brief, antibiotic resistance gene kanamycin (*aph*AI) flanked with arabinose inducible I-*Sce*I expression to remove kanamycin in the second recombination step was used as a positive selection marker. *aph*AI was first cloned into CXCL13s-pcDNA3 plasmids encodes CXCL13L1, L2 and L3 (Kindly provided by Prof. Pete Kaiser, The Roslin Institute and Royal (Dick) School of Veterinary Studies, University of Edinburgh, UK). Primers were designed to flank the kanamycin cassette with homologous sites (HR2) and unique *Bst*B1 restriction site, HR2 is a duplication of 40bp to allow removal

of *aphAI* in the second step of Red recombination mutagenesis (En passant mutagenesis) (section 2.2.2.8). PCR product and the vectors-plasmids which contain CXCL13s were digested with *Bst*B1 and ligated with T4 ligase, chemically transformed in compenant TOP10 *E.coli*., and plated O/N at 37 ^{o}C. on LB-amp-kan agar plates. Correct clones were confirmed by RFLP and sequencing.

2.2.2.8. Two-step Red-mediated recombination (En passant mutagenesis)

The Red-recombination system has the advantage to introduce of scarless modifications into large DNA constructs including long and short sequence insertions, deletions, point mutations or gene tagging, which cannot be done by other methods (134). This system was originated from *Enterobacteria* phage λ (lambdaphage) and consists of three major protein components; Exo, Beta and Gam proteins that can be activated at 42^{o}C. Linear ds DNA inserted to *E.coli*. will be degraded by the action of *E. coli* RecBCD helicase–nuclease complex, Gam protein of Red-recombination system inhibits this complex and maintains the inserted linear DNA. Beta protein protects the 5'-3' exonuclease action of homotrimer Exo protein, it also anneals the 3' single strand end produced from the linear DNA with complementary homologous sequences in the desired target, 30-50 bp is sufficient to allow an integration into replicating DNA in Red recombination system (135).

For scarless insertion or deletion of the inserted CXCL13s in MDV BAC by two-step Red-mediated recombination system, GS1783 *E.coli.* strain harbors RB1B-MDV BAC with temperature-inducible Red-recombination system, as well as arabinose induced the *I-SceI* homing endonuclease, and chloramphenicol resistance marker was used in this study. First, the transfer plasmids were used as templates and a PCR was amplified with primers that each possessed approximately 40 bp of homology to the desired target sequence in the BAC. Resulted linear DNA fragments were digested with *DpnI* enzyme to the shred original plasmid; the products were then purified by agarose gel extraction (section 2.2.1.7) and measured with Nanodrop 1000 (section 2.2.1.8). 100 ng of the clean linear PCR were electroporated into electrocompetent GS1783 *E.coli* harbor MDV BAC as described earlier (section 2.2.2.4). After electroporation, bacteria were resuspended in 1 mL SOC medium and incubated at 32°C for 2 h and finally spread on LB-cam-kan agar plates. Clones harbor the Kanamycin resistance selection marker (co-integrates) that grew on LB-cam-kan plates were screened for correct BAC backbone with RFLP (section 2.2.1.6) and compared with VNTI prediction.

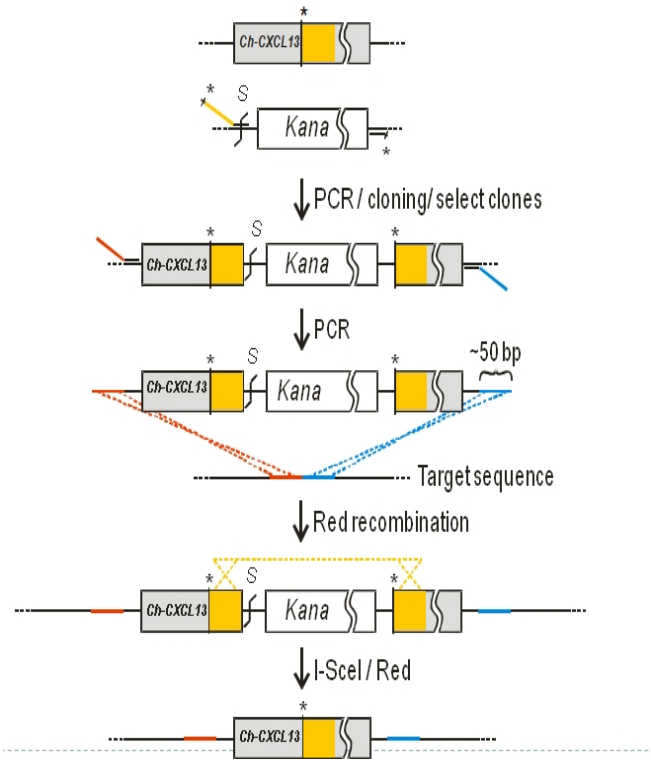

Figure 8: Schematic representation of the two-step Red-mediated recombination procedure
Two-steps Red recombination was used for insertion of CXCL13s into MDV-BAC. First, the kanamycin cassette obtained by a PCR from pEP Kans S contains *I-SecI* (S) with homologous sites (yellow box) is cloned into the single restriction site (*) of the CXCL13s-pcDNA3 plasmids. After PCR amplification with primers that add homologous flanks to desired location in BAC (red and blue lines), the first recombination step is performed in which the PCR product is integrated into the genome. The following process, resolution, is identical to what was previously described for sequence deletion. Adapted from Tischer et al. (135).

For scarless removal of the positive selection marker in the second Red-recombination step (Resolution), correct co-integrates were grown for 3h in 2 mL of LB medium with kanamycin and chloramphenicol at 32°C with shaking at 220 rpm. To induce the expression of *I-SceI*, 2ml of LB-cam containing 1% arabinose- were added to the culture and incubated for 2 h at 32°C with shaking at 220 rpm, cultures were then transferred to a 42°C water bath shaker (150 rpm) for 30 min to activate the Red-recombination system for a second recombination step between duplicated sequences **(Figure 8, shown in yellow)**, subsequently, bacteria were grown for another 3 h at 32°C with shaking, and 100 µl of $1*10^{-5}$ dilutions were spread on LB-cam-1% arabinose agar plates, incubated for 48h at 32°C. Replica plating was used to detect colonies that the Red-recombination second step had occurred (kanamycin-sensitive), these colonies (final mutants) were screened for correct BAC backbone with RFLP and sequencing PCR with primers cover the target sequence.

2.2.2.9. Preparation of bacterial stocks for long term storage

Bacterial clones contain correctly sequenced plasmids or BACs were stored in glycerol at -80 °C A single bacterial colony was used to inoculate 3 ml of LB medium containing the appropriate antibiotic. Cultures were incubated O/N at 32°C or 37°C with shaking at 220 rpm in the bacterial incubator, 500 µl of culture were mixed with 500 µl sterile SOB 50% glycerol in a 1.6 ml cryovial and stored at -80°C.

2.2.3. Virological and cell culture methods

2.2.3.1. MDV reconstitution from BAC DNA

Reconstitution of recombinant viruses was done by transfection of chicken embryo cells (CECs) prepared centrally in our lab as described previously (92). Briefly, 5 x 10^5 fresh CECs were seeded into one well of a 6-well plate with 2 ml of Minimum essential Medium Eagle (MEM) (Biochrom AG, Berlin) supplemented with 10%FBS-1% P/S and incubated O/N at 37 ºC and 5 % CO2 in humidified air cell culture incubator. Meanwhile, a 0.5-2 µg BAC DNA was diluted to 50 µl final volume with sterile 10 mM Tris (pH 7.5) in a polyethylene tube, 388 µl sterile Millipore water were added and mixed by gentel shaking to dissolve the DNA. A 62 µl 2 M $CaCl_2$ were added slowly in drops while gentle mixing. The transfection mixture was incubated O/N at 4ºC. Next day, a 500 µl ice-cold 2x HBS were added in a drop wise to the transfection mixture to form a calcium phosphate-DNA coprecipitate and incubated at RT for 15 min in the dark. Meanwhile, CECs were 80% confluent and ready to be transfected, media was removed and the cells were washed with 2ml PBS, supplemted with 500 µl fresh CECs growing media. After 15 min incubation time, 500 µl of the transfection mixture were added to each well, gently mixed and incubated at 37°C. After 3 h the transfection mixture was removed and the cells were washed with 2 ml PBS. Glycerol shock was then performed with 1.5 ml 1x HBS-15% glycerol for 2 min at RT. Cells were gently washed with 2 ml PBS, incubated in 2 ml MEM growing media until became complete confluent, after the cells were washed and shifted to maintenance MEM.

2.2.3.2. Virus stock and titration

After six days of transfection, plaques forming mutants were passaged three to five times to reach 70% infected cells prior to storage. Infected cells were washed two times with PBS, detached by 0.05% trypsin for 5 min at 37°C and aliquoted in 8% DMSO-MEM into 1.6 ml cryopreservation vials (1 ml of cell suspension per tube) and placed in a freezing container (Mr. Frosty, ThermoScientific) at -80°C O/N. The freezing container was filled with isopropyl alcohol to ensure the 1°C/min-cooling rate required for successful cryopreservation of cells. Finally, vials were stored in liquid nitrogen until use.

For viral titration, one vial from each aliquoted viral stock was thawed at 37°C in water bath for one min. and serially diluted to10^{-4} in MEM growing media. Dilutions of 10^{-2} to 10^{-4} were used to infect fresh 10^6 CECs plated in 6-wells with growing MEM in duplicate. Serum concentration was decreased and media was changed to maintenance MEM once confluency was reached. After 6 days, cells were washed with 2ml PBS, fixed with 2ml 90% ice-cold acetone for 10 min at -20ºC and then air dried for immunofluorescence staining.

2.2.3.3. Immunofluorescence Assay (IFA)

Indirect IF staining was used to visualize viral plaques in infected CECs. The cells were washed three times with 2ml PBS, fixed with 2ml 90% ice-cold acetone for 10 min at -20ºC and then air dried. Subsequently, for reducing non-specific binding; cells were blocked by 1% FBS diluted in PBS buffer for 1hr. After removing the blocking solution, the cells were stained with anti-US2-MDV chicken serum (1:5000) diluted in PBS 1% FBS for 1hr. The staining solution was aspirated and the cells were washed 3times for 5min with PBS. Goat anti-chicken Alexa Fluor 488 (1:1000) was used as secondary antibody for 30 min, then the cells were washed as describe earlier. Plaques were then counted and imaged with Zeiss AxioVert S100 fluorescence microscope.

2.2.3.4. Plaques size assay

10^6 fresh CECs were infected with 100 plaque forming unit (pfu). The cells were fixed 6 days later and IFA was performed as described above. Images of stained plaques from each virus were collected at 100x magnification and measured using the program Image J 1.48v (http://rsbweb.nih.gov/ij/), at least, 45 images were taken for each mutant and wild-type per experiment. Plaques area was converted to diameter according to the following equation:

$$Diameter = 2 * SQRT(area/3.14159)$$

Diameters then were normalized in comparison to wild type. All experiments described were performed at least three times in an independent fashion.

2.2.3.5. Growth Kinetics

Multi-step growth kinetic assays were performed simultaneously with the plaque size assays for all mutants. 10^6 CECs were infected with 100 pfu 10^6 fresh CECs in each well of a 6 well plate. One well was titrated daily for 6 days and IFA was performed as described above.

2.2.3.6. Baculovirus Expression Vector System (BEVS) and Insect Cell Culture

The baculovirus expression system (BEVS) (Invitrogen) is a convenient and versatile eukaryotic system for recombinant protein expression, which provides correct folding of recombinant protein as well as disulfide bond formation and other post-translational modifications (53). Expressed recombinant protein will most likely exhibit the proper biological activity and function (20). BEVS was used in this study for expression of recombinant chCXCL13s proteins in *Spodoptera frugiperda* (*Sf9*) insect cells *in vitro*. This system is based on the replacement of nonessential region of the wild-type baculoviral genome with the desired gene via homologous recombination. Transfection of *SF9* with the

recombinant transfer vector (Bacmid) containing the cloned gene allow producing infectious recombined baculovirus that contains the gene to be expressed under the control of the polyhedrin promoter. The production of the recombinant protein is achieved by infection of fresh insect cell cultures with the resultant recombinant virus **(Figure 9).**

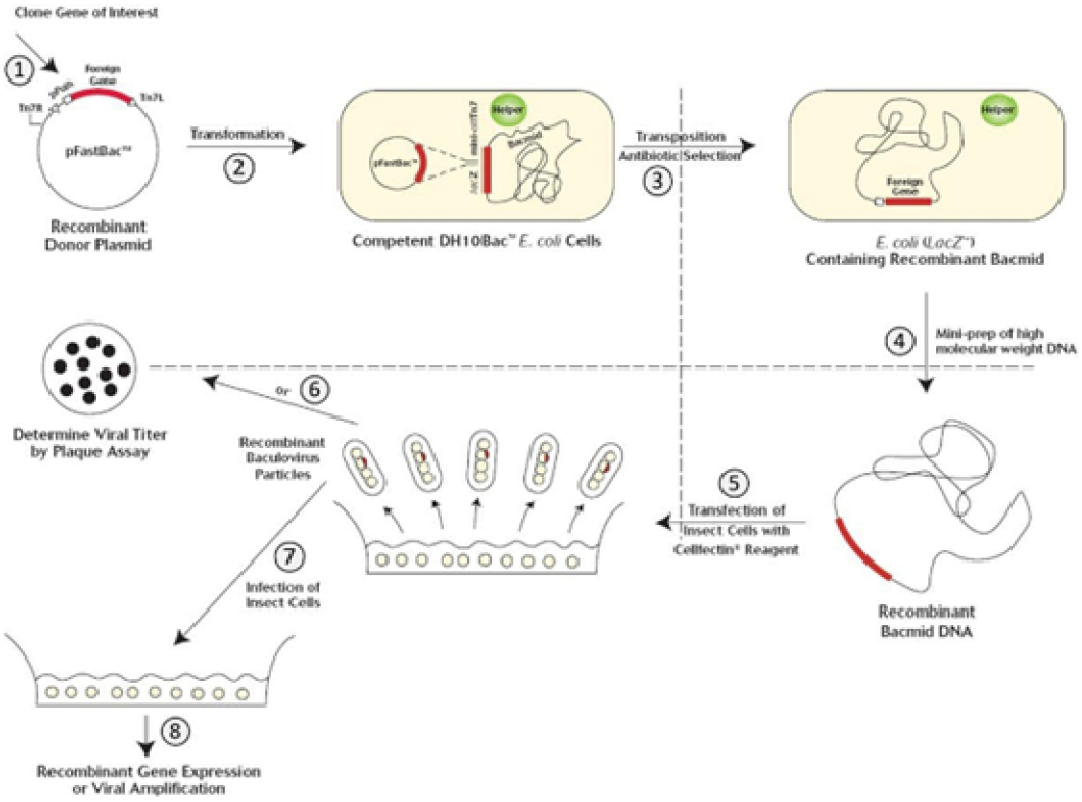

Figure 9: The baculovirus expression system (BEVS)

Chicken CXCL13s were cloned pFastBac11 (1), the recombinant plasmid is then transformed into DH10Bac competent *E.coli*. (2), containing a baculovirus shuttle vector (bacmid). Site-specific transposition then occurs between the two plasmids (3) in the presence of transposition proteins Tn7 transposase. CXCL13 was inserted into the Tn7 recombination site in the LacZ gene of the parent bacmid, and successful transpositions can be recognized by disruption of LacZ gene expression, yielding white bacterial colonies in the presence of X-gal. The recombinant bacmid DNA then isolated (4) and transfected into Sf9 insect cells (5). After five days, recombinant baculovirus can be isolated and tittered by a plaque assay (6) or used for infection of insect cells (7) for viral amplification and protein expression (8) (Invitrogen 2004).

2.2.3.7. Generation of transfer plasmid and recombinant bacmid

The transfer plasmid FC-His-pFastBac11 (Kindly provided by O. Negrete and B. Lee, (University of California Los Angeles, Los Angeles, CA) was generated by cloning of

fragment constant (FC) region of human immunoglobulin G (IgG) with a C-terminal His-Tag at the CopI site of pFastBac11 (Invitrogen) **(Figure 10B)** was used in BEVS to generated transfer plasmids for expression of chicken CXCL13 (L1, L2, and L3).

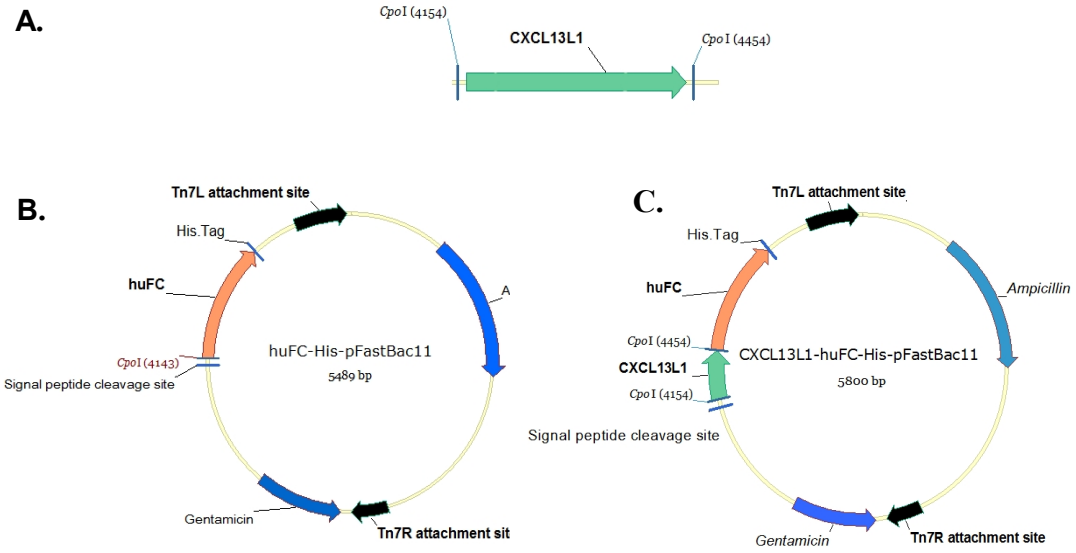

Figure 10: Constructions of cCXCL13L1 transfer plasmid for BEVS

A. The signal peptide of cCXCL13 was predicted as described above, PCR product with *Cpo*I restriction sites added to 5` and 3` to cCXCL13s, **B**. the transfer plasmid pFastBac11-FC-His and PCR product were digested with *CopI* and ligated to generate (**C**) cCXCL13L1-huFC-His-pFastBac11. The C-terminal His tag was denoted. Main futures of the transfer plasmid were denoted.

Briefly, the location of signal peptide cleavage site in chCXCL13L1, chCXCL13L2 and chCXCL13L3 was predicted using SignalP 4.1 serve (http://www.cbs.dtu.dk/services/SignalP/) (100), PCRs were amplified from pcDNA3.1 plasmids contain cDNA of chCXCL13L1, chCXCL13L2 or chCXCL13L3 (Kindly provided by Prof. Pete Kaiser, The Roslin Institute and Royal (Dick) School of Veterinary Studies, University of Edinburgh, UK) with primers where *Cop*I restriction sites flank each gene (without signal peptide sequence) **(Figure 10A)**, this allows chCXCL13s to be secreted with a C-terminal FC, followed by His tag (CXCL13-FC-His) depend on pFastBac11 promoter and signal peptide cleavage site. PCR products and FC-His-pFastBac11 were digested by *Cpo*I, ligated with T4 ligase, transformed into TOP10 *E.coli.*, and plated on LB-kan-gent-tet agar plates at 37 ºC. Correct clones were confirmed by RFLP and sequencing. The FC-His-pFastBac11 allowed construction of transfer plasmid for baculovirus expressing the FC-His only as a control protein.

To generate recombinant bacmids, 5ng of correct clones of transfer plasmids were chemically transformed into 100µl competent DH10Bac *E.coli.* contain baculovirus bacmid (Invitrogen) according to the manufacturer's protocol, the transformed cells were then diluted 1:1000 and 100µl were spread on LB/kan-gen-tet for selection of DH10Bac transformants, as well as 100µg/ml X-gal for blue/white selection of colonies that contain recombinant bacmid and the plates were incubated at 37 ºC for 24 h. White colonies were picked and correct clones were confirmed by sequencing PCR using the forward and reverse primers mentioned in manufacturer's protocol (Invitrogen). The recombinant bacmid DNA was isolated with QIAGEN Plasmid Midi Kit (2.2.2.6) and DNA concentration was measured by Nanodrop 1000 (section2.2.1.8).

2.2.3.8. Transfection of *SF9* by recombinant bacmid

Sf9 insect cells were transfected with recombinant bacmids by Fugene HD (Promega, Mannheim) according to the manufacturer's instructions. Briefly, $1x10^6$ *Sf9* insect cells were seeded in 6-well plates in 2 mL Insectomed SF express® (Biochrom AG, Berlin) at 27 ºC for one hour to adhere. 2µg bacmid DNA were mixed with 8.2µL Fugene HD (Promega, Mannheim) and diluted in 150 µl Opti-MEM® I Reduced Serum Media (Invitrogen) for 30 min. The adhered cells were washed in 1 mL Insectomed SF express® media, and 2 mL media were added followed by the transfection mixture. The cells were incubated at 27ºC for five days to recover first virus amplification.

2.2.3.9. Production and amplification of recombinant baculovirus protein

For further baculovirus amplification, $6x10^6$ *Sf9* cells were seeded with 10 ml media in 100mm dishes and incubated at RT for 1h for adherence. The cells were infected with 1 ml of the first virus passage for three days, the media was then collected and centrifuged at 14000 rpm at 4 ºC for 10 min, and the supernatant termed the second virus passage was recovered. For large scale production of recombinant protein for further purification, $1x10^6$ *Sf9* cells/ml was cultured in 50 ml media in sterile 1000 ml Erlenmeyer flask and incubated at 27 ºC with shaking at 120 rpm for three days. Cells were then infected with viral stocks grown for further three days and harvested then by centrifugation at 1400 rpm for 10 min at 4 ºC.

2.2.3.10. The mammalian recombinant protein expression system

Transient expression systems in mammalian cells provide a valuable method for producing of recombinant proteins (130). The system characterized by rapid, good protein yields and low coast compared to other expression systems. Also, human embryonic kidney cells

(HEK) are adapted to grow and reaching high densities even with serum-free media. HEK cells can be transiently transfected using an inexpensive transfection reagent such as polyethyleneimine (PEI). The mammalian expression system maintains post-translational modifications. It is valuable for producing proteins that require complex folding.

Chicken CXCL13s and RB1B-vIL-8 were first cloned into the expression plasmid pCR3.1 (Invitrogen). For CXCL13s, PCRs were amplified from pcDNA3.1 plasmids contain cDNA of cCXCL13L1, cCXCL13L2 or cCXCL13L3 (Kindly provided by Prof. Pete Kaiser, The Roslin Institute and Royal (Dick) School of Veterinary Studies, University of Edinburgh, UK), with primers where *HindIII* and *SalI* restriction sites flank each gene. CXCL13s were cloned in the frame with huFc to be secreted with a C-terminal Fc. For vIL-8, the PCR was amplified from RB1B BAC DNA, flanked with *HindIII* and *SalI* restriction sites and cloned as in CXCL13. PCR products and pCR3.1 vector were digested with *HindIII* and *SalI*, ligated with T4 ligase, transformed into TOP10 E.coli., and plated on LB-Amp agar plates at 37 ºC. Correct clones were confirmed by colony PCR, RFLP and sequencing.

2.2.3.11. **Transient transfection mediated by PEI**

HEK 293 cells were transfected using polyethyleneimine (PEI). Stock solutions of PEI were prepared using a 25 kDa linear PEI (Polysciences, Warrington, PA, USA) in a concentration of 1 mg/ mL. PEI solution was prepared in water, neutralized with HCl, sterilized by filtration (0.22 µm) and aliquoted and stored at –70 °C.

Briefly, for each well of a 6-well plate, 3 µg DNA of cloned pCR3.1 expression plasmids were diluted in 200 µl Opti-MEM® I Reduced Serum media (Invitrogen) and mixed with 9 µl PEI for 30 min. The transfection mixture was added to 80% confluence cells and incubated O/N at 37 ºC and 5 % CO2 in humidified air cell culture incubator.

2.3. Protein analyses

2.3.1.1. **Western Blot**

For immunological detection of proteins from viral infected CECs or from recombinant-baculovirus infected *SF9*, samples were mixed with 6x SDS sample loading buffer and denaturated by heating at 95 ºC for 5 min. Proteins were then separated on either 12 or 15 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) under reducing conditions, then electrophoretically transferred onto a polyvinylidene difluoride (PVDF) membranes using semi-dry transfer apparatus in transfer buffer at 1.2 mA/ cm^2 for 40 min. To minimize non-specific interaction of the antibody, the membrane was incubated for 1 h in blocking solution (5 % non-fat milk powder in TPBS) at 4 ºC with shaking (120rpm). All

primary antibody dilutions were 1:1000 in 1X TBS, 0.03% Tween-20 (0.03% TBST) unless mentioned otherwise. Incubation with primary antibody was performed for O/N s at 4 ºC with shaking (120rpm). After incubation, the membrane was washed with 0.03% TBST once briefly and three times for 5 min with shaking at RT. Subsequently, the membrane was incubated for 1 h at 4 ºC with shaking (120rpm) with goat anti-mouse IgG or goat anti-rabbit IgG horseradish peroxidase (HRP) conjugated secondary antibodies at a 1:5,000 dilution in the blocking solution. The membrane was again washed as described above. HRP-antibodies bound to the target protein were detected by means of the ECL Prime Western Blotting Detection Reagent (Amersham Biosciences), detection of antigen-antibody complexes on the membrane was performed by chemiluminescence under a Chemi-Smart 5100 detection system (PeqLab).

2.3.1.2. Detection of secreted vIL8 and cCXCL13s from viral infected CECs

To detect the expression and secretion of RB1B-vIL-8 and chicken CXCL13s from recombinant viruses; $1x10^6$ CECs were infected with 1000 pfu of vRB1B, vΔMetvIL-8, vTK-CXCL13L1, L2, L3 or TK-CXCL13-revertant viruses (rev). Supernatants from infected cells were harvested at 6 dpi and 500 µl were concentrated using an Amicon Ultra-0.5 centrifugal filter unit with a 10-kDa cutoff (Millipore). Secreted chemokines were analyzed by western blotting as described above. For detection of vIL-8, CXCL13-His and gC as a loading control; polyclonal rabbit anti-vIL-8 antibody, rabbit anti-His Tag and rabbit anti-MDV-gC were used as primary antibodies respectively described early. After washing, the membrane was incubated with HRP-conjugated goat anti-rabbit IgG antibody and detected as described above

2.3.1.3. Detection of recombinant proteins in the baculovirus expression system

For detection of recombinant chicken CXCL13-His tagged protein, supernatant from recombinant-baculovirus infected SF9 were harvested by centrifugation at 14000 rpm at 4 ºC for 10 min. samples were separated by 12% SDS-PAGE and analyzed by western blotting as described above. 1:1000 polyclonal rabbit anti-His Tag was used as described early. After washing, the membrane was incubated with HRP-conjugated goat anti-rabbit IgG antibody and detected as described early.

2.3.1.4. Detection of recombinant proteins in the mammalian expression system

For detection of recombinant chicken vIL-8-huFc and CXCL13-huFC tagged protein, supernatant from HEK293 transfect with pCR3.1 cloned plasmid for each chemokine were harvested two days post transfection. 500 µl were concentrated using an 10% (v/v) trichloroacetic acid, vortexed and incubated on ice for 30 min. After incubation the precipitated proteins were pelleted by centrifuging at 10,000 g for 15 min. The pellets were then washed two times with 1 ml 90% ice-cold acetone. After washing the pellets were air dried and resuspended 50 µl of 6x SDS sample loading buffer. 25 µl were separated by 12% SDS-PAGE and analyzed by western blotting as described above. 1:1000 polyclonal goat anti-huFc or rabbit anti-vIL-8 were used as described early. After washing, the membrane was incubated with HRP-conjugated goat anti-rabbit IgG antibody and detected as described early.

2.3.1.5. Coomassie stain

Proteins were separated by 12 % SDS–PAGE as described above; the gel was then washed with Millipore water for 10 min and transferred to Coomassie staining solution for O/N on orbital shaker at RT, the staining solution was removed and the stained gel was washed first with Millipore water to wash out the remnants of the dye and then destained with destaining solution on orbital shaker. The destaining solution was changed repeatedly to decrease the washing time and to gain a more transparent gel. Pictures were taken by Chemi-Smart 5100 detection system (PeqLab).

2.3.1.6. Purification of His-Tagged cCXCL13 by Batch Method

The recombinant chicken CXCL13s proteins expressed by BEVS were purified through the nickel batch purification method. Briefly, 500 ml of infected *SF9* supernatant were mixed with an equal volume of equilibration buffer. 3 ml of HisPur™ Ni-NTA Resin (Thermo Scientific) were sedimented by centrifugation at 700 × g for 2 min, the supernatant was then discarded and the resin beads were washed with equilibration buffer followed by centrifugation for 2 min at 700 × g. The washed beads were then mixed with equilibrated supernatant on an orbital shaker for 1h at RT. To remove nonspecific proteins that weakly binds; resin beads were washed 3 to 5 times with washing buffer until the supernatant became clear. The His-Tag proteins were eluted from the beads with 6ml elution buffer. Three consecutive elutions were performed and the collected fractions were labeled as E1, E2, and E3, respectively. Sample from each step was analyzed by Coomassie staining and western blot.

2.3.1.7. **Recombinant protein dialysis**

Recombinant protein was further purified by dialysis in order to remove imidazole, salts, and for proper folding of protein. Dialysis was carried out against PBS, using VISKING® Dialysis Tubing (MWCO 12000 – 14000, Serva, Heidelberg). The sample was dialyzed two times 1 h each and the third time for O/N at 4°C. Dialysis was carried out in 200 times the volume of the sample.

2.4. Binding assay and chemotactic assays

The binding assay of CXC chemokines to clonal CXCR5+ HEK293 cells were done by Dr. Sonja Härtle (Department für Veterinärwissenschaften Lehrstuhl für Tierphysiologie, Ludwig-Maximilians-Universität, München, Germany).

To identify the binding of vIL-8 to chicken CXCR5; approximately 5 x 10^5 of stably transfected CXCR5-Flag cells HEK 293 (100% positive) were mixed with untransfected HEK293. This mixing allow to identification of a positive and negative staining. Cells were mixed with 100µl supernatant of vIL8-huFc, cCXCL13 (L1, L2, and L3)-huFc. The huFc was mixed with untransfect cells supernant as the negative control. The cells were then incubated on ice for 30 min with occasional gentle agitation. Samples were then washed three times with 1ml ice-cold 1% FBS-PBS, followed with incubated for 20 min with 100 µl of 1:1,000 goat anti-hu IgG-Alexa Fluor® 647 conjugate secondary antibody. Cells were then washed twice and re-suspended in 200µl FACS buffer. Transfected and stained cells were analyzed using a BD FACSCanto™II. Acquisition parameters were established using untransfected cells, unstained or cells stained with secondary antibody only.

2.5. *In vivo* experiment

Recombinant viruses that grew *in vitro* and secreted CXCL13 chemokine were evaluated *in vivo* to investigate if chicken CXCL13s can complement vIL-8 knockout MDV in pathogenesis and tumor formation.

2.5.1.1. **Viruses and animal infection**

Eggs from specific pathogen free (SPF) layer chickens (VALO®, Lohmann Tierzucht, Germany) were incubated and hatched in the Institute of Virology, Freie Universität Berlin. Hatched chicks were then neck-tagged with individual numbers and randomly distributed and maintained in different groups under isolated conditions in the animal facilities of the Institute of Virology, Freie Universität Berlin. All animal experimentations were approved and carried out following the institutional guidelines for animal care. Animals received commercial layer feed and water ad libidum.

At the day of hatching (day 0), animals were blindly infected intra-abdominally with 1,000 pfu of vRB1B, vΔMetvIL-8, vTK-CXCL13L1, vTK-CXCL13L2, vTK-CXCL13L3, and vTK-CXCL13L1-rev. Ten naïve animals were kept uninfected in the same cage with infected animals to investigate the virus transmission via the natural route of infection. Viruses were titrated again on CECs after infection to determine the minimum infectious dose per chicken. Dead animals in the first week post-infection were considered as early mortality and excluded from the experiment analysis. Chickens were monitored daily for clinical symptoms of MD. Animals were examined for tumors lesions post mortem once clinical symptoms were evident or at the experiment termination (91 dpi). Stability of the CXCL13 in Mini-F and vIL8 start codon mutation was confirmed by DNA sequencing derived from blood at 28 dpi.

2.5.1.2. **Quantification of MDV genome copies in chicken whole blood**

The ability of recombinants mutants to grow and replicate *in vivo* was evaluated by quantification of MDV genome copies in whole blood during the animal experiment. 40 µl blood were collected from the wing vein and mixed with 20 µl 100mM EDTA in 1 ml deep-well plates. Blood was collected from 8 birds in each group at different time points; for infected animals at 4, 7, 10, 14, 21, 28, and 91 dpi., and for contact animals at 21, 28, 35, 42 and termination day. DNA was isolated by E-Z96 96-well blood DNA isolation kit (Omega Biotek, USA) according to manufacturer`s instructions.

Quantitative PCR (qPCR) was performed using primers and probe specific for the MDV-ICP4 gene, normalized to total DNA loading for each sample with primers and probe specific for the housekeeping gene iNOS. For generation of the standard curves in qPCR assays; DNA template from RB1B-BAC encodes ICP4 gene or plasmid encodes iNOS gene were used to calculate copy number using Thermo Fisher Scientific web-based calculator: (https://www.thermofisher.com/de/de/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/dna-copy-number-calculator.html). The standard curve generated for each gene ranged from 10-10^7 copies/µl, and one as non-template control (NTC).

Each qPCR reaction contained TaqMan Fast Universal Master Mix (Applied Biosystems), 10 µl DNA, 25 pmol of each gene-specific primer and 10 pmol of the gene-specific probe in a 20 µl total volume. One reaction was left as NTC to ensure no DNA template contamination. Thermal cycling conditions were as follows: 95°C for 20 s, followed by 40 cycles at 95°C for 3 s and 60°C for 30 s. Threshold was set to start at the exponential phase of the reaction, the cycle threshold value (CT) is the cycle number at which the fluorescence generated within a reaction crosses the fluorescence threshold, plotted to the total copies of each

standard dilution in duplicate The coefficient of regression (R^2) was always near to 0.99 for standard curves. Using the standard curve generated for each gene; the numbers of copies for ICP4 and iNOS were determined by using CT for each sample. CT value of 36 indicated no specific amplification of the target DNA and a value of 0 copies in the sample was used in the analysis. All qPCR assays were performed in an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Inc.) and the results were analyzed using Sequence Detection 7500 System (v2.0.6). MDV genome copies were determined by dividing the number of ICP4 copies by the number of iNOS copies, multiplied by 1,000,000 and the values were expressed as the number of MDV copies per 10^6 cells.

3. Results

3.1. Evolutionary and functional characterization of vIL-8

3.1.1. vIL-8 is an ELR- CXC chemokine, shares a higher homology to CXCL13 than CXCL8

To determine the true orthologue of vIL-8, we performed analyses of all known human and chicken CXC chemokines amino acid. Amino acid sequence analysis revealed that vIL-8 possesses the characteristic criteria of CXC chemokines, including an N-terminal signal peptide and spacing of the four conserved cysteines residues. The first two conserved cysteines residues located at position 42 and 44, and the two remaining cysteines at position 69 and 89. In addition, a conserved proline is also presents in the amino acid all analyzed chemokines **(Figure 11)**.

Figure 11: Sequence comparison of MDV and chicken CXC chemokines

Multiple alignments of cCXC chemokines with reference to MDV-vIL-8. Amino acid alignments were performed using the ClustalW W method of Vector NTI Advance™ 9.1. Signal peptides are underlined. Conservative ELR motifs are indicated under the red line, conservative cysteine residues are highlighted in black. Conservative proline residues are also found among all chemokines. Other conservative residues, similar and weakly similar in gray, green and blue respectively. Alignment gaps are indicated by dashes. Sequences are identified by their systemic names GenBank accession numbers listed in **Table 3**.

vIL-8 possesses a DKR motif at N-terminus of the first conserved cysteine residue in vIL-8 and classified as ELR- CXC chemokine **(Figure 11)**. ELR motif is conserved in all inflammatory CXCL8 like (cCXCL1 (GROa), cCXCL8L1 (K60), and CXCL8L2 (CAF)). Like vIL-8, CXCL12, CXCL13L1, L2, L3 and CXCL14 do not contain the ELR motif and thus belong to homeostatic ELR- CXC chemokine. Both vIL-8 and CXCL13L1 have a long C-terminus than the other CXC chemokines.

The amino acid similarity analysis showed that vIL-8 has only a limited homology to the three inflammatory chicken CXC chemokine cCXCL1 (GROa), cCXCL8L1 (K60), and CXCL8L2 (CAF) (21.1%, 28.8% and 31.1%) respectively **(Table 8)**. Of all the chicken CXC chemokines evaluated, cCXCL13L1, cCXCL13L2, and cCXCL13L3 showed the highest similarity to vIL-8 with 53.8%, 33.0%, and 36.1%, respectively. vIL-8 also shares high sequence similarity with CXCL13L1 in the signal peptide region, and the 60-amino-acid stretch following the conserved first two cysteine residues. The C-terminus 18 amino acids of the protein are the most diverged. cCXCL13L2 and cCXCL13L3 sequences are very closely related **(Table 8)**.

Table 8: Amino acid similarity between chicken and viral CXC chemokines

Percentage identities of chicken (c) and vIL-8 CXC chemokine amino acid demonstrated by pairwise comparisons by MEGA6 program (129)

	CXCL1 (GROa)	CXCL8L1 (K60)	CXCL8L2 (cCAF)	CXCL12	CXCL13L1	CXCL13L2	CXCL13L3	CXCL14
CXCL1(GROa)	100.0							
CXCL8L1 (K60)	38.9	100.0						
CXCL8L2 (cCAF)	36.6	68.6	100.0					
CXCL12	19.5	23.9	26.4	100.0				
CXCL13L1	24.2	28.8	32.0	23.9	100.0			
CXCL13L2	25.3	29.3	31.6	16.7	41.0	100.0		
CXCL13L3	28.6	33.0	40.0	19.0	38.1	53.1	100.0	
CXCL14	22.6	24.2	26.7	24.4	21.3	21.6	26.2	100.0
vIL-8	21.1	28.8	31.1	22.7	**53.8**	**33.0**	**36.1**	19.1

3.1.2. **vIL-8 is the orthologue of chicken CXCL13L1**

The phylogenetic trees were constructed using the Neighbor-joining method and Maximum likelihood method within MEGA 6.0 **(Figure 12**). The results showed that vIL-8 clustered with orthologues group of human and chicken CXCL13, directly linked to cCXCL13L1. vIL-8 has a relatively low homology to human and chicken CXCL8, not linked to human or chicken CXCL8.

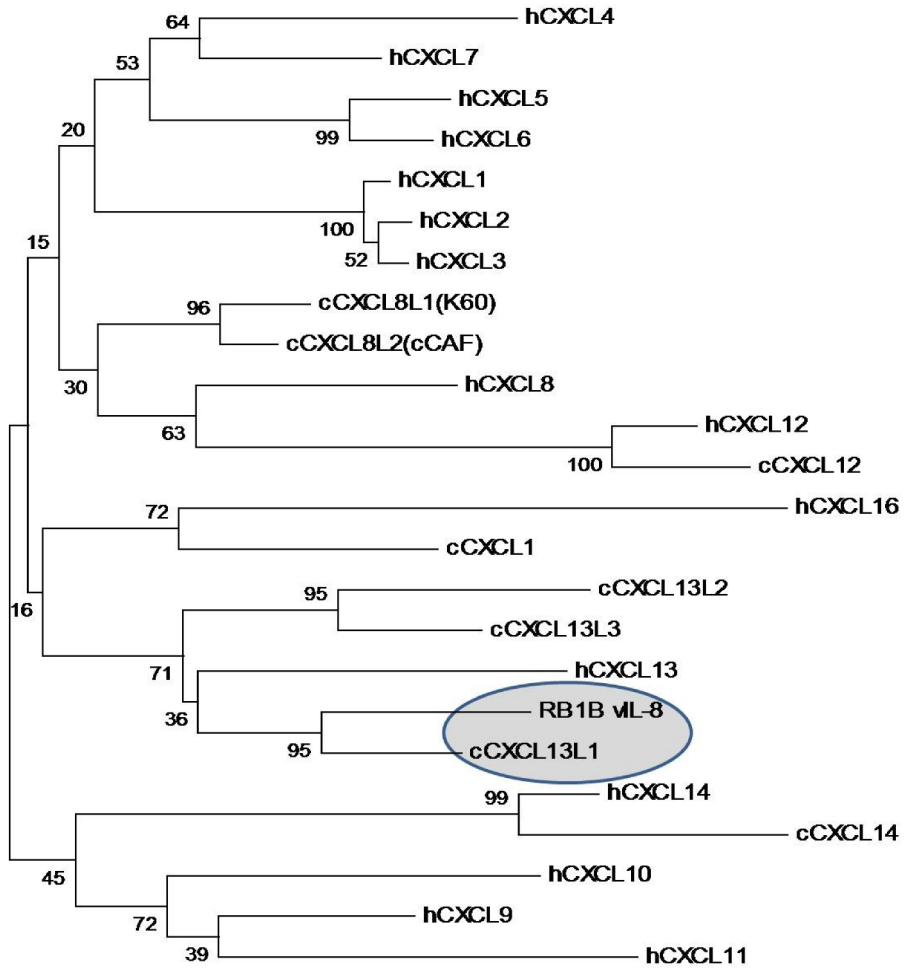

Figure 12: Phylogenetic tree of amino acid sequences of CXC chemokines of human (h), chicken (c), and MDV (vIL-8)

Phylogenetic trees were constructed using full length amino acid sequences of vIL8, chicken and human CXC chemokine with Neighbor-joining method and Maximum likelihood method within MEGA 6.0. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Human (h) and chicken (c) chemokines are indicated and common name in brackets. The scale bar reflects the horizontal distance at which amino acid sequences differ by 10% between two sequences

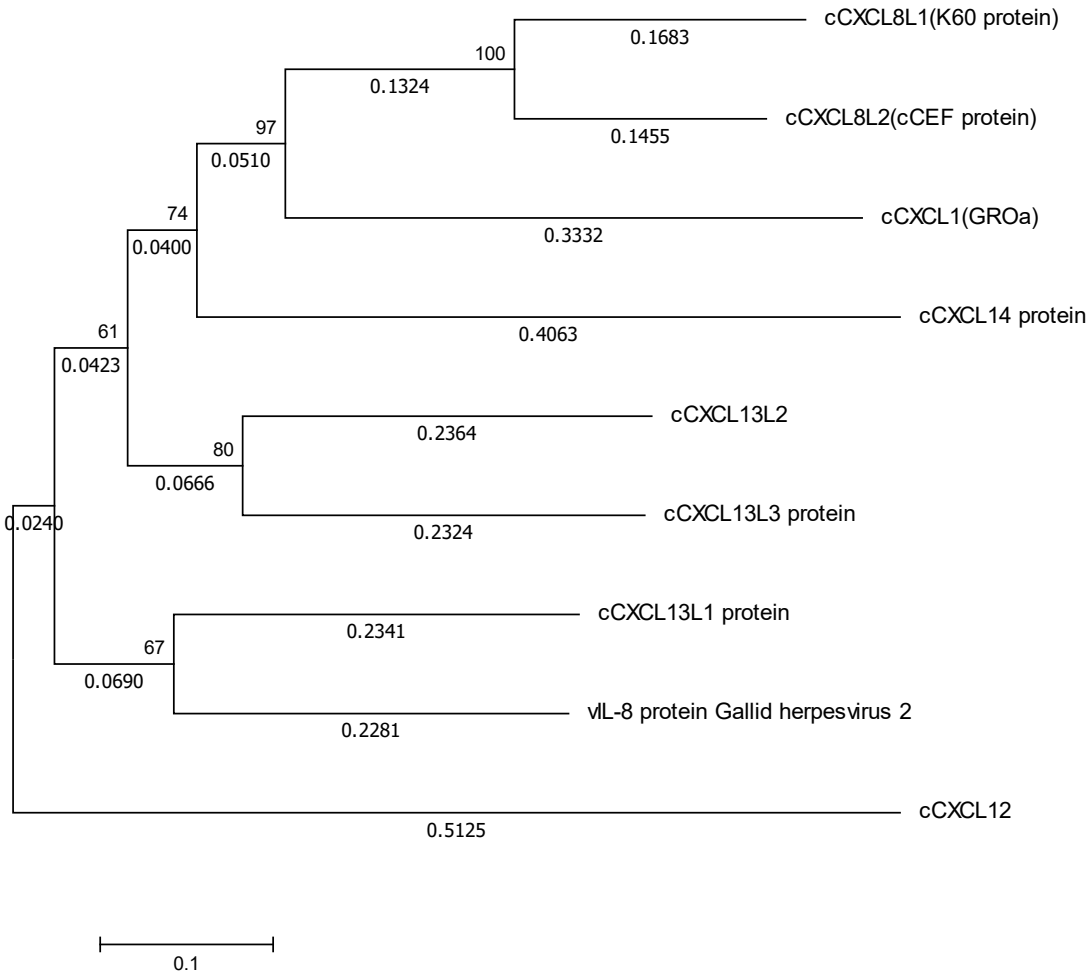

Figure 13: Evolutionary relationships of vIL-8 and chicken CXC chemokine

Phylogenetic trees were constructed using full length amino acid sequences with Neighbor-joining method and Maximum likelihood method within MEGA 6.0. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches

These findings indicate that CXCL13 is the closest relative of the viral chemokine vIL-8, suggesting that cCXCL13L1 is the ancestory gene of vIL-8.

We confirmed our finding with using blast-based searches, and three available homology search softwares, INPARANOID (http://inparanoid.sbc.su.se/cgi-bin/blast_search.cgi) (108), OrthoDB software (http://www.orthodb.org/software) (66), and OMA (http://www.omabrowser.org) (5, 6) refer vIL-8 as the orthologue of chicken CXCL13L1.

3.2. vIL-8 binds chicken CXCR5

3.2.1. Recombinant expression and purification

3.2.1.1. Baculovirus Expression Vector System (BEVS)

Bioinformatics analysis showed that CXCL13L1 is the vIL-8 orthologue. To investigate a potential interaction between vIL-8 and cCXCL13s with chicken CXCR5, recombinant vIL-8 fused to the human IgG-Fc (huFc) were previously generated in our lab using the baculovirus expression system (BEVS) (43). I used the BEVS to generate the three variants of cCXCL13 (L1, L2 and L3) in the same way and as described in methodology (section 2.2.3.9). The recombinant proteins were purified as described previously (section 2.3.1.6). The purified proteins were analyzed by western blot and shown in **Figure 14A and B.**

The results confirm the expected molecular weight for huFC of 25 kDa, vIL-8 at of 42 kDa, CXCL13L2 and CXCL13L3 of about 38 kDa. In addition to the expected band, a band for cleaved off huFc appeared with vIL-8 and CXCL13L2. Unfortunately the size of CXCL13L1 did not match the prediction of 38 kDa **(Figure 14C)**, an unexpected protein band of approximately 15 kDa present in the eluted fraction of CXCL13L1 after recombinant protein purification. This unexpected band protein of CXCL13L1 may reflect cleavage or digested form of chemokine, or there is a splice variant exists in the *SF9.* In addition, the yield was very low compared to the other recombinant proteins **(Figure 14C).** Another band of 25 kDa represent a cleaved off huFc also appeared. The two bands on western blot analysis of

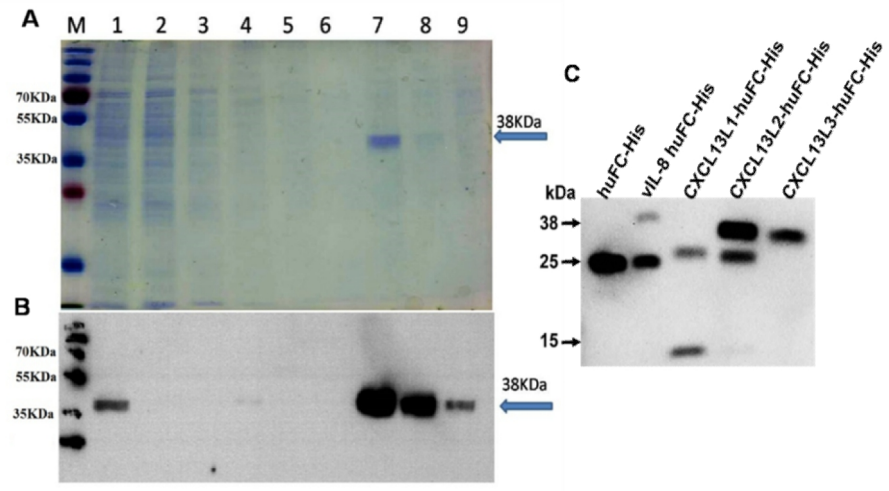

Figure 14: Expression and purification of cCXCL13s by BEVS

SDS-PAGE (0.1% SDS-12% polyacrylamide, Coomassie blue stained and Western blot analysis of cCXCL13L3 protein expressed in Sf9 cells. (**A**) SDS-PAGE. M; Protein Marker, 1 infected *Sf9* cells equilibrated supernatant, 3, 4, 5 and 6; are four consecutive washes, 7,8 and 9; are three consecutive elutions). (**B**) Western blot analysis using the rabbit anti-His tag monoclonal antibody. (**C**) Western blot analysis of purified protein. Membrane stained with anti-His Tag antibody.

CXCL13L1 (25 KDa and 15 KDa) indicated that the protein produced by BEVS was not correctly folded and couldn't be used for functional characterization of CXCL13L1. For further *in vitro* characterization, functional recombinant vIL-8 and CXCL13s proteins were produced using a mammalian expression system.

3.2.1.2. Recombinant cCXCL13s and vIL-8 can be produced by mammalian expression system

CXCL13L1 produced using BEVS couldn't be used for *in vitro* assays in term of yield and folding. To obtain adequate yield of correctly folded functional recombinant chicken CXCL13s and vIL-8 proteins, I used the mammalian expression system using HEK293 cells. The mammalian expression system using Human Embryonic Kidney 293 (HEK 293) cell line has been extensively used for expression of recombinant proteins. This system is capable of carrying out most of the post-translational folding and processing required generating functional, mature protein from mammalian and non-mammalian genes. The vIL-8 and CXCL13s expression plasmids were cloned and transfected as described in methodology (sections 2.2.3.10 and 2.2.3.11). Culture supernatants containing secreted chemokines were collected and analyzed for protein expression by western blot **(Figure 15)**. The total amount of secreted chemokines was determined by ELISA performed by Dr. Sonja Härtle in LUM (data not shown).

Western blot analysis confirmed the correct size of the monomeric, non-glycosylated form of CXCL13L1, L2 and L3 of approximately 38 kDa (**Figure 15A**). An additional smaller band of observed for CXCL13L1, most likely represents a degradation product of the chemokine. The size of huFC and vIL-8 were larger than expected, likely due to glycosylation as observed previously. vIL-8 was also detected by western blot using a vIL-8 specific rabbit polyclonal antibody. Another band was present below the major band of vIL-8 which most likely represents the degradation form of vIL-8 (**Figure 15B**).

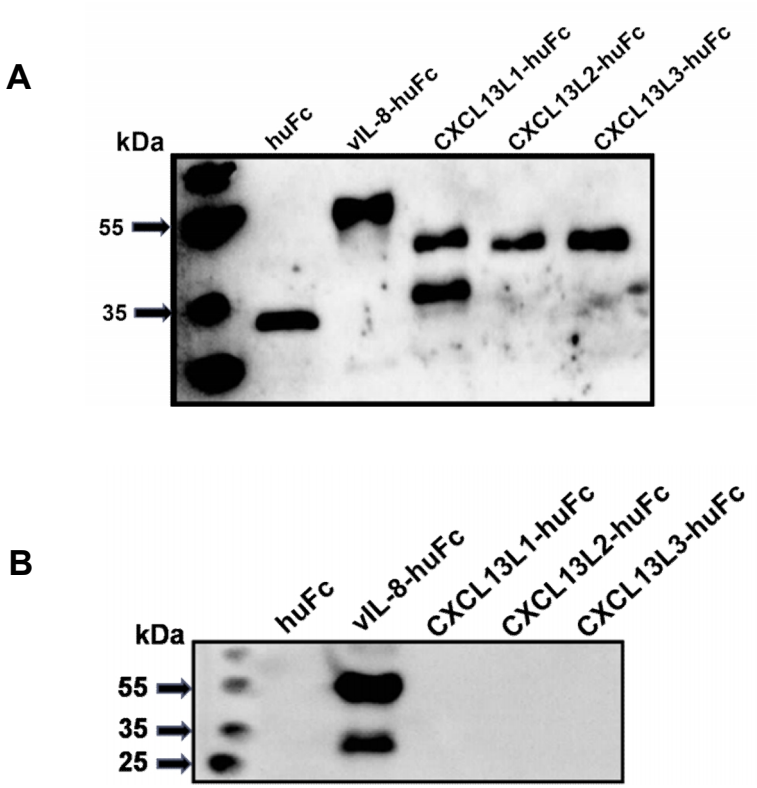

Figure 15: Western blot analysis of vIL-8 and cCXCL13s produced by eukaryotic expression system

Western blot analysis HEK293 cells supernatant transfected with indicated expression plasmid. (**A**) Target proteins were detected using goat anti-huFC. antibody or (**B**) stained with rabbit anti-MDV/vIL-8. as described in methodology (2.3.1.4)

3.2.2. vIL-8 and cCXCL13L1 bind chicken CXCR5

3.2.2.1. vIL-8 binds chicken B cells that express cCXCR5

CXCL13, also known as B lymphocyte chemoattractant (BLC), binds and attract B cells by binding its exclusive receptor CXCR5. In chicken, CXCL13 was previously known as DT40 CXC chemokine due to it binds DT40 chicken B cell line. To investigate if vIL-8 is the functional orthologues to CXCL13, I analyzed the binding of vIL-8 to primary bursal B cells and DT40 chicken B lymphocyte cell line. To correlate the binding of vIL-8 with CXCR5 expression; chicken CXCR5 receptor was first detected on the chicken primary bursal and DT40 B lymphocytes cell line using mouse anti-cCXCR5 antibody (kindly provided by Dr. Sonja Härtle, LUM). In both types of chicken B cells, CXCR5 was highly expressed **(Figure 16A and Figure 17A)**. Binding assay was performed on the CXCR5+ DT40 B lymphocyte. The results showed that both vIL-8 and CXCL13L1 bind to CXCR5+ B cells. CXCL13L2 showed a weak binding, while CXCL13L3 did not bid DT40 cell line **(Figure 16.B)**

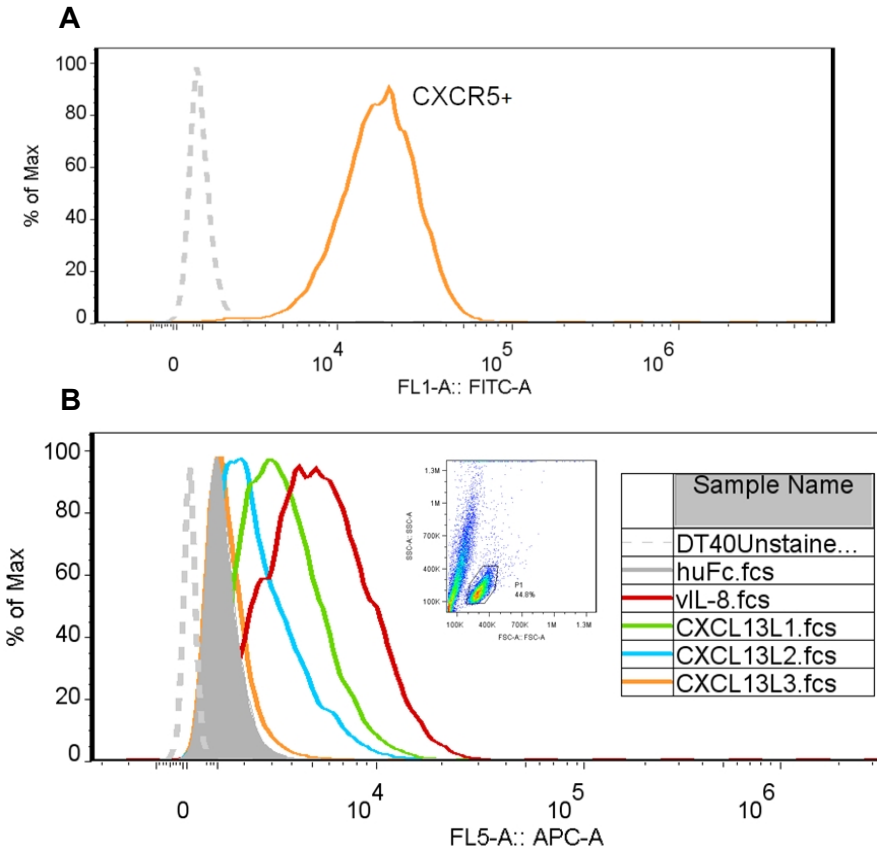

Figure 16: vIL-8 binds CXCR5+ chicken DT40 B lymphocyte cell line

(**A**)Cell surface expression of cCXCR5 on DT40 chicken B lymphocytes cell line Image for anti-cCXCR5 antibody using Alexa 488-fluorochrome-conjugated antibodies. (**B**) binding of vIL-8-huFC, CXCL13s-huFc to DTB40 chicken B lymphocytes cell line. Samples gatting were shown. Image for anti-huFC antibody using Alexa 647-fluorochrome-conjugated.

As for DT40 B cell line, both CXCL13L1 and vIL-8 bind to primary B-cells **(Figure 17B)**. No binding was detected for CXCL13L2 and CXCL13L3. Our data clearly show that both vIL-8 and CXCL13L1 bind different chicken B cells types which express CXCR5 receptor.

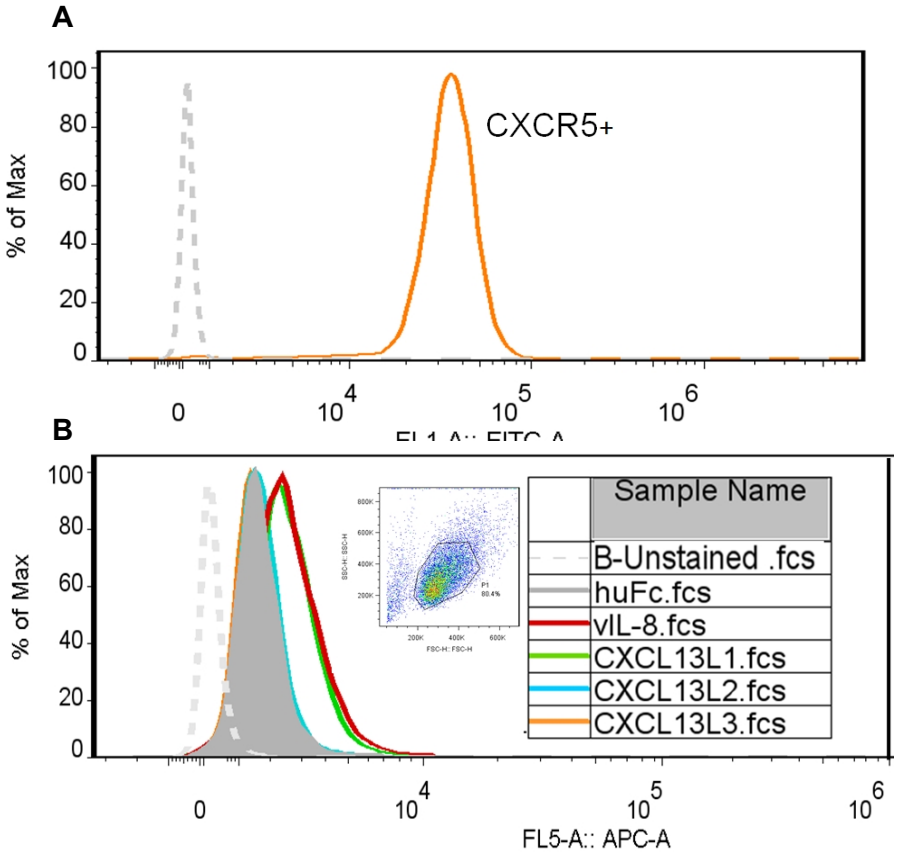

Figure 17: vIL-8 binds chicken CXCR5+ chicken primary bursal B cells

(**A**) Cell surface expression of cCXCR5 on primary bursal chichen B cells. Image for anti-cCXCR5 antibody using Alexa 488-fluorochrome-conjugated antibodies. (**B**) binding of vIL-8-huFC, CXCL13s-huFc to primary chicken bursal B cells. Samples gatting were shown. Image for anti-huFC antibody using Alexa 647-fluorochrome-conjugated.

3.2.2.2. vIL-8 and cCXCL13L1 binds chicken CXCR5

To determine if vIL-8 binding to B-cells is mediated through CXCR5, chicken CXCR5 was stably expressed on the surface of HEK293 cell line, which naturally lacks chicken CXCR5. Clonal CXCR5+ HEK293 cells were mixed with equal number of normal HEK293 to facilitate a better differentiation between positive and negative binding. Staining with monoclonal mouse anti-chicken CXCR5 was discriminate CXCR5 positive and negative HEK293 cells **(Figure 18A).** The binding assay revealed that both vIL-8 and CXCL13L1 bind CXCR5 at comparable levels. CXCL13L2 show non-specific binding with CXCR5 negative cells and CXCL13L3 showed only minimal binding **(Figure 18)**. These findings indicate that CXCR5 is the receptor of vIL-8 and that CXCL13L1 is the functional orthologue of the viral chemokine.

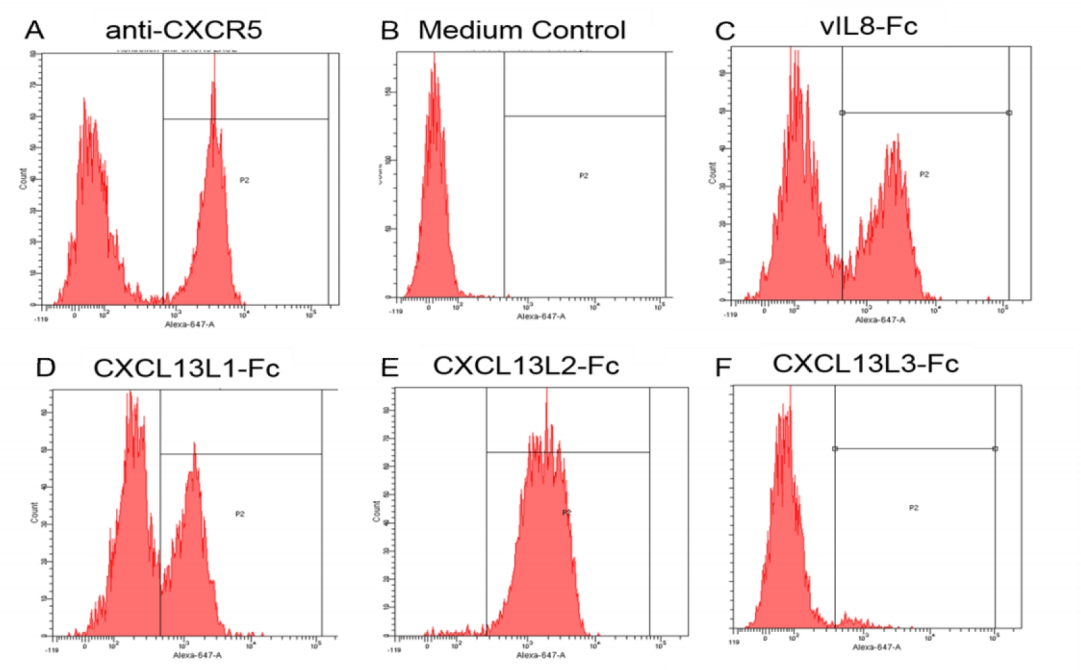

Figure 18: vIL-8 and cCXCL13L1 binding toHEK293T cells expressing cCXCR5

Clonal CXCR5+ and CXCR- HEK293T cells were mixed to get better discrimination between positive and negative cells. (**A**) Cell surface expression of CXCR5 by HEK293T cells as determined by FACS using the mouse anti-chicken CXCR5 antibody. Cells were incubated with indicated chemokine. After washing cells were stained with Alexa fluorochrome 647-conjugated goat anti-huFc (Figure provided from Dr. Sonja Härtle)

3.3. Role of vIL-8 in MDV replication and pathogenesis

3.3.1. Generation and *in vitro* characterization of recombinant viruses

3.3.1.1. Insertion of cCXCL13L1 at the C-terminus of vIL-8 does allow chemokine secretion

To determine if chicken CXCL13 (L1, L2 or L3) can complement the role of vIL-8 in MDV pathogenesis; I generated recombinant viruses using the bacterial artificial chromosome (BAC) system of the very virulent RB1B strain. The original vIL-8 locus was preferred for insertion of chicken CXCL13 to maintain the kinetics of chemokine expression. *En-Passant* mutagenesis was used to insert C-terminal His-tagged CXCL13L1 into the C-terminus of vIL-8. The recombinant virus was generated and successfully reconstructed, however, no secreted protein was detected (data not shown), indicated that this spliced region interfere with CXCL13L1 synthesis and secretion. The complex splicing in this region between vIL-8, meq and other neighboring genes like RLOF4 may affect the protein expression and virus pathogenicity. Also, acceptor sites in CXCL13 were predicted (http://www.fruitfly.org/cgi-

bin/seq_tools/splice.pl), which may interfere with the splicing in this region and CXCL13 synthesis and secretion. Therefore, another location in MDV BAC was targeted to allow virus replication and chemokines secretion.

3.3.1.2. Insertion of cCXCL13 at the unique long region impaired virus replication *in vitro*

To obtain recombinant viruses that can replicate and secrete chicken CXCL13, the C-terminal His-tagged CXCL13s were inserted into the C-terminus of UL45 of ΔMet-vIL-8-RB1B under the control of the cytomegalovirus immediate early promoter (pCMV) (CMV-CXCL13L1-UL45, CMV-CXCL13L2-UL45 and CMV-CXCL13L3-UL45). In addition, revertant viruses were generated by removal of inserted chicken chemokines (CMV-CXCL13L (1, 2 and 3)-UL45-rev). Mutants were analyzed by RFLP using three enzymes and by sequencing PCR of the target locus. RFLP of CMV-CXCL13L1-UL45 is shown as an example in **Figure 19**.

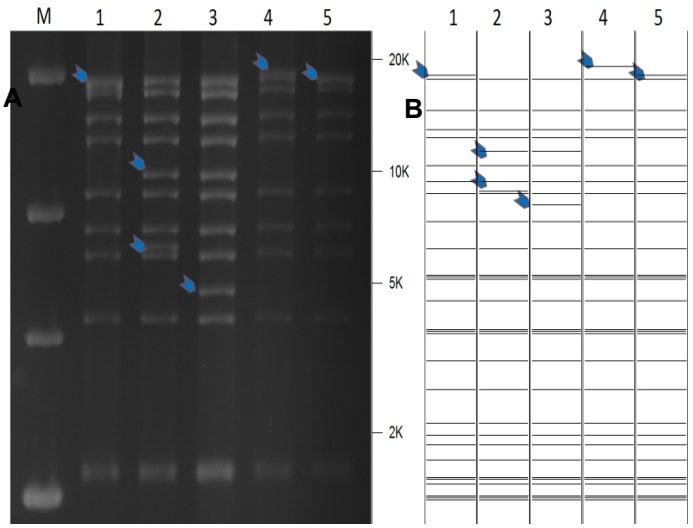

Figure 19: RFLP patte rns of CMV -CXCL13s insertion in MDV -UL45 BAC
(A) Patterns obtained by restriction enzyme BamHI run through 0.8% agarose gels overnight at 65 V. (B) Vector NTI 9.1 prediction RFLP patterns are shown with size markers from 2 to 20kb (M). (1) ΔMetvIL-8, (2) CMV-CXCL13L1-cointegrate-UL45, (3) CMV-CXCL13L1-UL45, (4) revertant-cointegrate (5) ΔMet-vIL8-revertant.

Intriguingly, recombinant viruses vCMV-CXCL13L (1, 2 and 3) were not able to replicate upon virus reconstitution in chicken embryo cells (CECs) by calcium transfection. Only individually infected cells were observed, while the revertants mutants replicated and formed plaques comparable to wild type and parental virus. Plaque size assay results showed the significant difference (Kruskal-Wallis test, $p < 0.0001$) between recombinant L1 expressing viruses compared to wild type, parent virus, and revertants **(Figure 20)**. Therefore, the

insertion of CMV-CXCL13 at the C-terminal region of the UL45 inhibit *in vitro* virus replication.

To address if this inhibition effect of virus growth is due to the influence of CMV-CXCL13 or the target locus, we targeted other location in the unique long region, between UL35 and UL36 that had been previously used for the expression of transgenes. However, the insertion in this region resulted in highly impaired viruses that could not replicate like wild type virus. The comparison of non-viable mutants was made by plaque size assays directly post-transfection **(Figure 20)**

3.3.1.3. Insertion of TK-CXCL13s in mini-F permits *in vitro* chemokine secretion and virus replication

Insertion of CXCL13 in unique long regions of MDV BAC resulted in severely-impaired viruses. To exclude the effect of the insertion locus, the minimal fertility factor replicon (mini-F) was targeted for the insertion of CXCL13 in MDV BAC. Mini-F is essential for maintaining BAC sequence replication in *E.coli.,* but is dispensable for viral replication *in vitro* and *in vivo*. The *E.coli.* xanthine-guanine phosphoribosyltransferase gene (xgpt) in mini-F was replaced with CXCL13s under the effect of pCMV. Recombinant BAC constructs confirmed by RFLP and sequencing of PCR product (data not shown). Recombinant mutants of CMV-CXCL13L2 and CMV-CXCL13L3 were successfully reconstituted by calcium transfection in CECs, replicated and form plaques, however, CMV-CXCL13L1 was unable to reconstitute as no plaques have seen after transfection. These finding indicate that the insertion of CXCL13 under strong pCMV promoter is site independent for CXCL13L1, however, mini-F location permit CXCL13L2 and CXCL13L3 recombinant viruses replication under effect of CMV strong promoter.

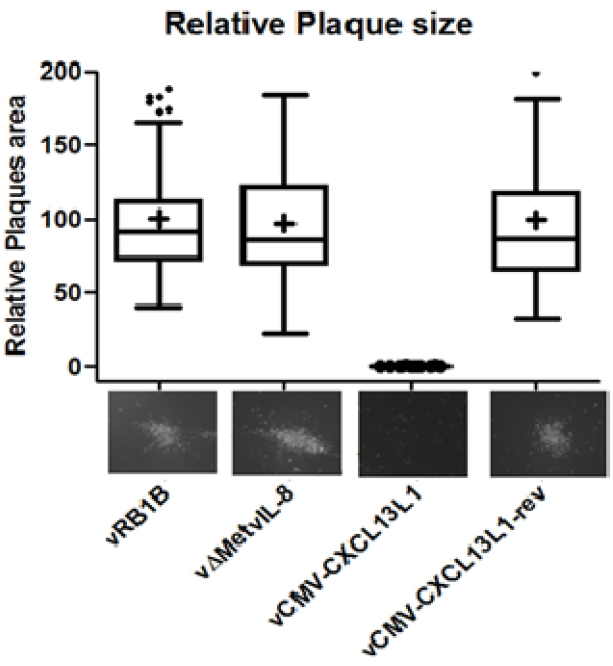

Figure 20: Plaques formation and relative plaques area of CMV-CXCL13 in UL45
vRB1B, vΔMetvIL-8, vCMV-CXCL13L1-UL45 and revertant. (A, B, C, and D) show a representative plaque for each virus underneath. (E) The relative plaque area induced by each virus was determined; Box-plot show median line and 95% distribution of plaques area, Means are shown as`+`. Values that were significant differences (Kruskal-Wallis test, $p < 0.0001$) are indicated with an asterisk (*). Results of vCMV-CXCL13L1-UL45 are representative to CMV-CXCL13L2 and L3 in both UL45 and UL35 (data not shown).

To solve the apparent problem of overexpression, I constructed recombinants viruses in which pCMV were replaced by a weaker thymidine kinase promoter (TK-CXCL13L1, L2, or L3) in mini-F. Revertants viruses were also generated by the removal of CXCL13 (TK-CXCL13L1, L2, or L3- rev). The replacement of mini-F xgpt with CXCL13s as well as the removal of inserted CXCL13s was confirmed by RFLP and sequencing of PCR product using primers flanking the target site **(Figure 21)**.

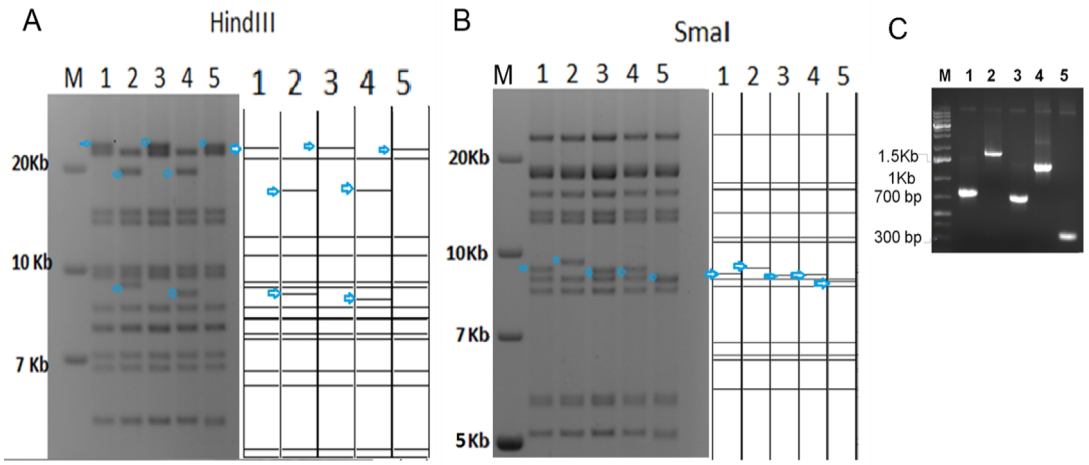

Figure 21: RFLP pattern of TK-CXCL13s insertion in MDV BAC-Mini-F

(**A**) RFLP patterns and Vector NTI prediction obtained by digestion of 2µg BAC-DNA with *Hind*III restriction enzyme (**B**) and *Sma*I. Gel run through 0.8% agarose gels overnight at 65 V. (**C**) Agarose gel of PCR products obtained by sequencing primers of target site for insertion of TK-CXCL13s by replacing xgpt gene in mini-F. M: DNA marker shows size from 2-20 kb, 1: ΔMetvIL-8, 2: TK-CXCL13L1-mini-F cointegrate, 3: TK-CXCL13L1-mini-F, 4: rev-TK-CXCL13L1-mini-F cointegrate, 5: rev-TK-CXCL13L1-mini-F.

Recombinant mutants were successfully reconstituted by calcium transfection in CECs, replicated and formed plaques in a manner comparable to wild-type and revertants viruses **(Figure 22A and B)**. Chicken CXCL13s in mini-F were successfully transcribed, translated and secreted driven by the TK promoter. The secreted proteins could be detected by western blot after concentration of supernatant of infected cells as described previously (section 2.3.1.2) **(Figure 22C)**. These results showed that replacing strong CMV promoter to a weaker TK promoter allow in vitro recombinant viruses replication and secretion of chicken CXCL13s.

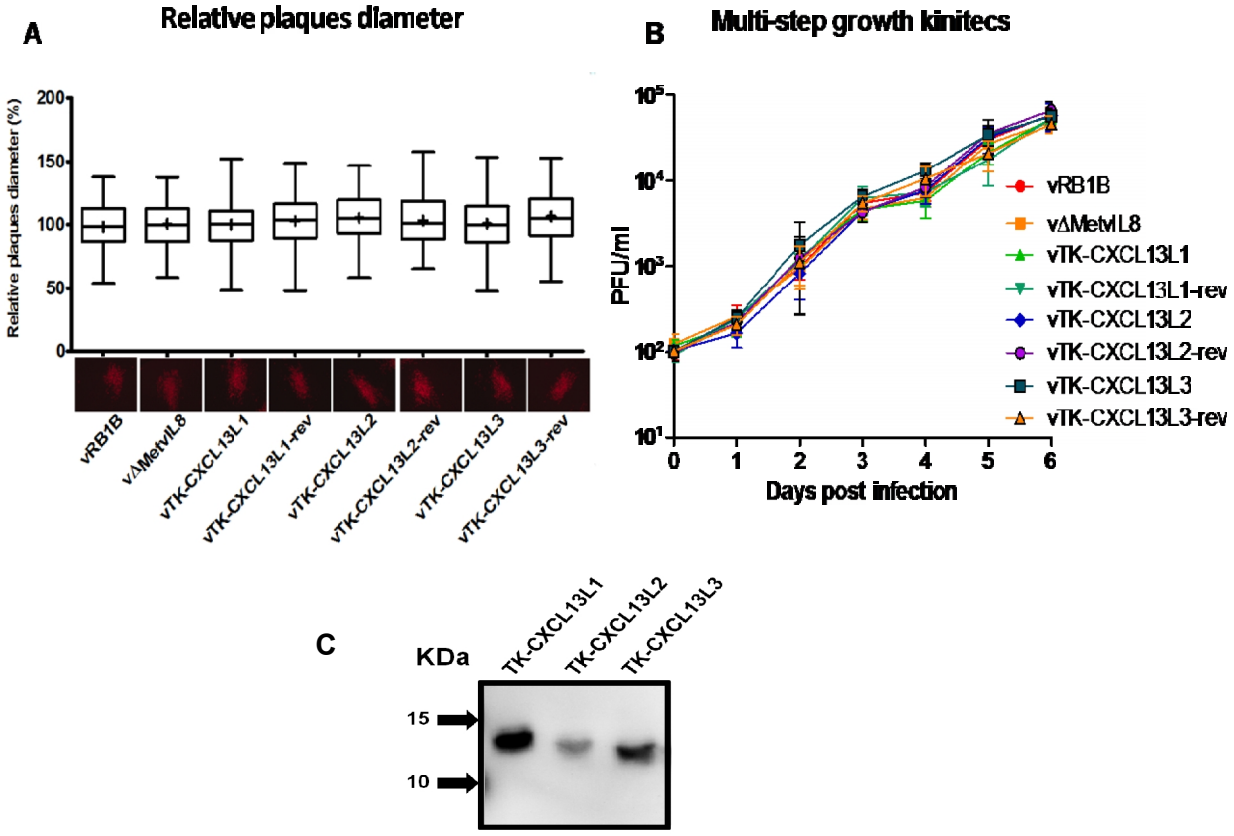

Figure 22: *In vitro* characterization of RB1B, ΔMetvIL-8, TK-CXCL13s-mini-F and TK-CXCL13s-mini-F-revertenants

(**A**) Images for at least 45 plaques were taken by fluorescence inverted microscope; the area was measured by Image J 1.48v software, converted to diameter and normalized to wild type. Plaque diameter in Box-plot shows median line and 95% distribution of plaques area. Means are presented as`+`. A representative plaque displayed underneath (x40). Results are the median of three independent experiments. (**B**) Multiple-step growth kinetics of the indicated viruses shown as mean with SEM (Kruskal-Wallis, $p<0.05$). (**C**) Western blot analysis detecting C-terminus His tagged CXCL13s in the concentrated supernatant of CECs infected with indicated viruses

3.4. *In vivo* animal experiment

The second aim of the study is to investigate if chicken CXCL13s particularly CXCL13L1 can compensate vIL-8 abrogation MDV in pathogenesis and tumor formation *in vivo*. An animal experiment was performed by infection of one-day-old chicks intra-abdominally with 1,000 pfu of vΔIRL-RB1B, vΔMetvIL-8 and vTK-CXCL13 (L1, L2 or L3)-mini-F. Contact animals were kept uninfected in the same cage with infected animals to investigate the transmission of the virus via the natural route of infection. The *in vivo* MDV replication assessed by quantification of the expression of MDV ICP4 gene and related cellular iNOS genes in the DNA isolated from whole blood of infected and contact animals at different time points as

described previously **(Figure 23A and B)** respectively. The results showed that MDV genome copies in infected animals with vΔMetvIL-8, TK-CXCL13L1 and CXCL13L3 were slightly reduced compared to those observed after infection with wild-type and TK-CXCL13L2. However, no significance differences in the lytic replication of all recombinant viruses were found in infected animals ($p < 0.05$) **(Figure 23A and B)**. Viral genome could be detected in contact animals of wild-type group at day 21dpi., continued to increase over time before declining after 42dpi. However, viral replication in contact animals of the other mutants was extremely low compared to wild type contact. None of the contacts of CXCL13L1 and CXCL13L3 was positive in qPCR. None of the vTK-CXCL13s mutants contact animals showed MD symptoms or lesions, indicated that the viruses were unable to spread and replicate in the contact animals. These finding showed that secreted chicken CXCL13s under TK promoter didn't complement vIL-8 abrogation.

Wild-type virus caused clinical signs in 50 % of infected birds (MD50) by 49 dpi while all in mutants couldn't reach MD50 until termination (91 dpi) **(Figure 23-C)**. At the final necropsy, the overall MD incidence for all recombinant viruses was significantly lower than wild-type and almost similar to the virus with abrogated vIL-8 (vΔMetvIL-8). 41.6% of contact animals in wild-type virus developed the disease, while the contact animals in other virus are negative for MD symptoms and lesions, indicate that the TK-CXCL13 has no effect and couldn't complement the function of vIL-8 abrogation MDV **(Table 9)**. At necropsy time, only one contact animal of vTK-CXCL13L1 has enlarged kidney. However, no clinical signs were observed in this bird, and the viral load was absent in this animals (as well as all tested animals in the group). Sequencing PCR of DNA isolated from the affected organ, confirmed that the recombinant viruses were correct and still harbored the CXCL13s.

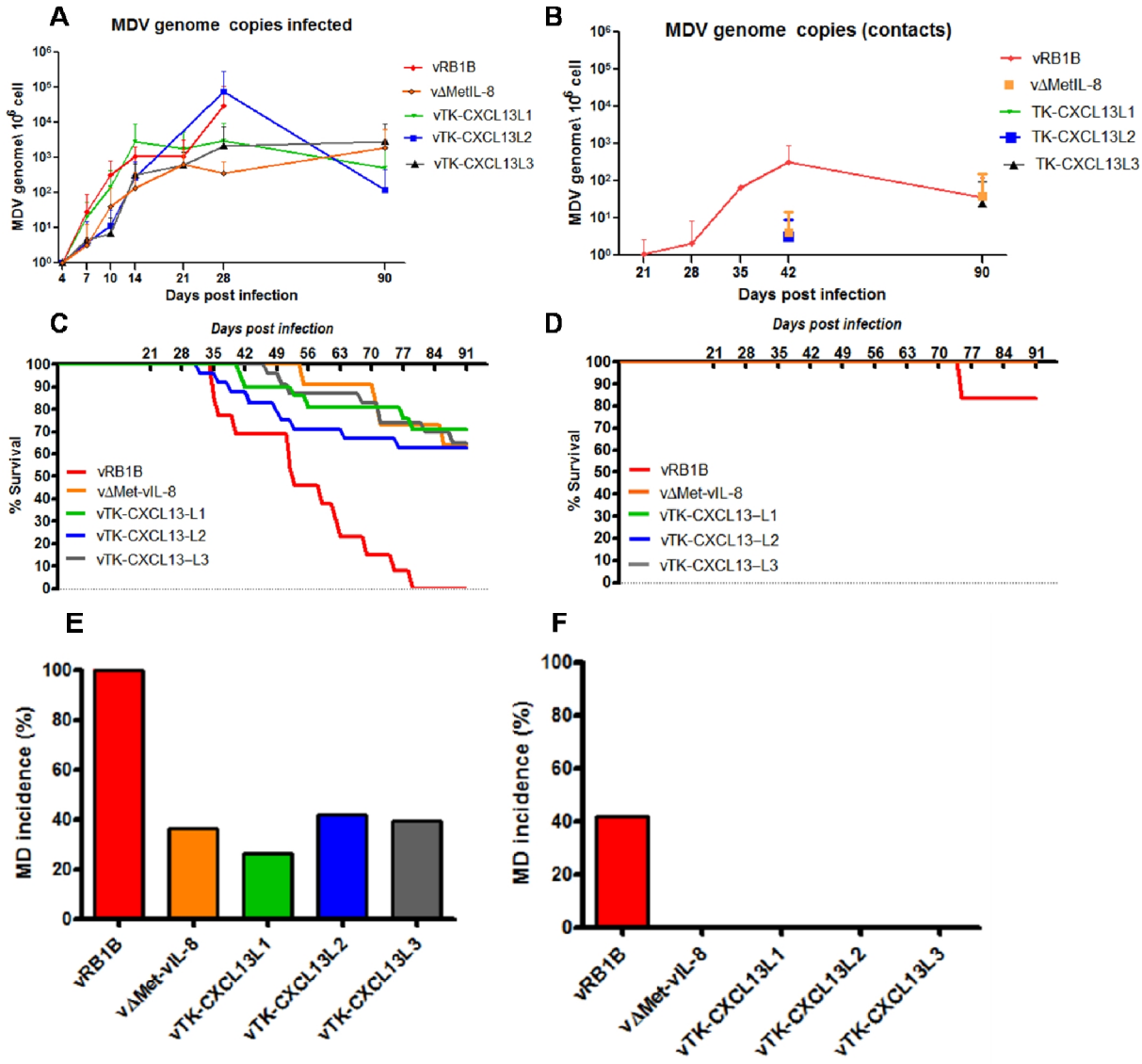

Figure 23: *In vivo* characterization of vTK-CXCL13-mini-F

One-day old chicks were infected intraperitonealy with 1000 pfu of indicated viruses. (**A-B**)Mean of MDV genome copies from blood samples of eight animals per group of infected (**A**) and contacts (**B**) of indicated virus determined by qPCR at different time points. (**C-D**) Survival analysis of infected (**C**) and contacts (**D**) chickens of indicated viruses. (**E-F**) Percentage of MD incidence in infected (**E**) chickens and contacts (**F**) with indicated viruses. Animals were checked daily and necropsies were performed on upon onset of clinical symptoms or at the time of experiment termination. MD incidence is shown in percent of animals per group of indicated virus.

Our bioinformatics analysis and *in vitro* binding assay clearly indicate that vIL-8 is a functional orthologue of chicken CXCL13L1. The *in vivo* results showed that CXCL13s couldn't compensate vIL-8 abrogation, which may be caused by variations in the expression kinetics between vIL-8 in its original locus and promoter with CXCL13 in mini-F under TK promoter.

Table 9: Animal experiment for detection of TK-CXCL13 MDV pathogenicity

Recombinant virus	MD incidence	
	Infected	**Contact**
vΔIRL-RB1B	100% (n=10)	41.6% (n=12)
vΔMetvIL-8	36.4% (n=11)	0% (n=13)
vTK-CXCL13L1	26.1 (n=25)	10% (n=10)*
vTK-CXCL13L2	41.6% (n=24)	0% (n=12)
vTK-CXCL13L3	39.13% (n=23)	0% (n=13)
*A bird with enlarge kidney, but no clinical signs and viral genome couldn't be detected in blood or from affected organ		

To investigate this assumption, I generated a recombinant virus by tagging the vIL-8-C-terminus with 6xHis tag to enable comparable secreted chemokines quantification with C-terminus His-tagged CXCL13. Secreted chemokines were collected from viral infected CECs, analyzed by western blot as described in methodology (section 2.3.1.2) and shown in **Figure 24**. The quantity vIL-8 secreted in its original location driven by its own promoter was much higher compared to CXCL13 under TK promoter in mini-F. This difference explains why CXCL13 could not complement the function of vIL-8 in the *in vivo* experiment.

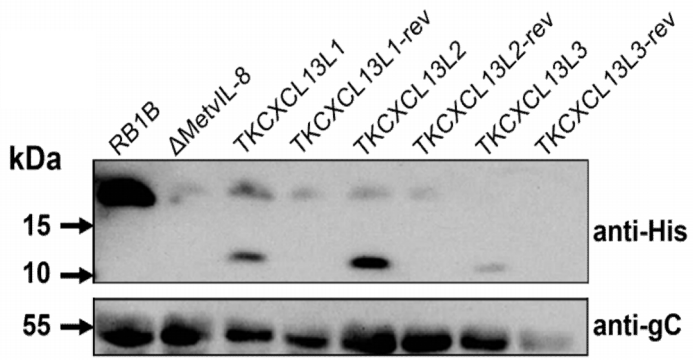

Figure 24: Quantification of viral secreted chemokines in recombinant viruses
Western blot analysis detecting C-terminus His tagged vIL-8 and CXCL13 (upper panel) or gC (lower panel) in the supernatant of CECs infected with indicated viruses

The difference between secreted vIL-8 in original locus and promoter with TK-CXCLs in the mini-F was also quantified using western blot image analysis. The relative amount of secreted chemokines was normalized to the relative amount of secreted vIL-8. The results showed that vIL-8 was secreted two times more than TK-CXCL13L1 and TK-CXCL13L2, while it was ten times more than TK-CXCL13L3. This finding point the differences in expression kinetics of chemokines that explain the compensation of TK-CXCL13s in mini-F of vIL-8 in MDV pathogenesis.

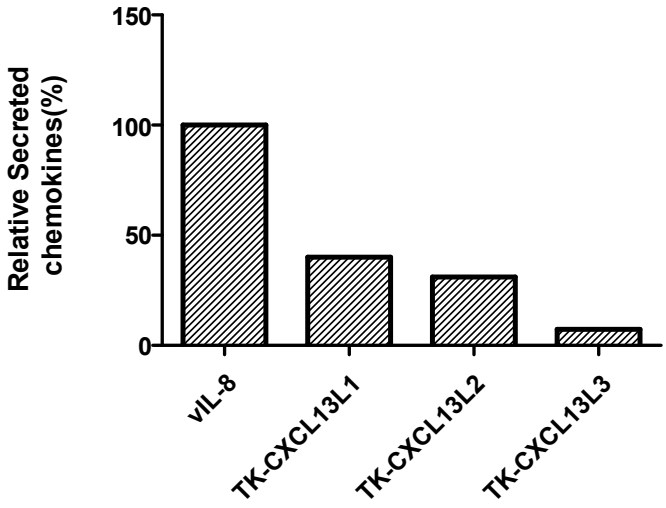

Figure 25: *In vitro* relative quantity of secreted vIL-8 and TK-CXCL13s
Western blot picture was analyzed using Image J software (115). The relative amount of chemokine represented by the peak areas plotted for anti-His stained chemokine of the mentioned lane in Figure 24, relative to the peak areas of the respective gC. The relative measures of TK-CXCL13s were then normalized to relative quantity of vIL-8 and represented in percentage.

4. Discussion

- **MDV vIL-8 is the functional orthologues of chicken CXCL13L1**

The first aim of the study was to determine the true orthologue of vIL-8 among all up to date described chicken CXC chemokine, with relation to human CXC. Orthologues genes defined as homologues genes acquired by a species during evolution process. In other words, orthologues genes are one particular gene in two related species (114). Correct detection of orthologue is a crucial task for precise prediction of the gene function in the related species, as well as to understand the biochemical pathways of these genes in different species. Furthermore, it allows the establishment of the correct taxonomy to facilitates the characterization of the target molecule (1).The differences between MDV-vIL-8 and chicken IL-8 (cCXCL8) at the gene and protein levels, as well as the type of cells recruited , suggested that vIL-8 is not an IL-8 orthologue.

The goal of this thesis was to identify the true cellular orthologue for vIL-8. Identification of orthologue genes can be performed basically by methods includes phylogenetic tree analysis of certain related genes, and large-scale blast-based method using orthologues detection softwares (108, 140). Phylogenetic tree analysis is a valuable tool to describe the evolutionary relations between individual genes (140). The release of the complete chicken genome enables annotation of several chicken chemokines and chemokine receptors genes (2, 36, 56). The blast-based method characterized by both high sensitivity and specificity provides a broad range of genes as well as a fast and accurate tool to find orthologue gene among available genome in different organisms (7, 140). Both approaches identify the orthologues and refer them to their most recent point of origin. Consistent with the previous report on the differences between vIL-8 and cCXCL8; phylogenic tree results showed that vIL-8 is not related to human and chicken CXCL8 counterparts, also supported with the differences in the gene (three exons for vIL-8 and four exons for cCXCL8) and protein similarity (36, 56) **(Table 8)**. The absence of ELR motif is consistent with a previous report showed that vIL-8 does not recruit heterophile (98), propose that vIL-8 did not bind to CXCR1, the receptor of cCXCL8 (72).

Online blast analysis of three softwares demonstrated that cCXCL13L1 (also known as B lymphocyte chemoattractant (BLC) or B cell-attracting chemokine 1 (BCA-1) is the ancestor of vIL-8, which is also directly linked in the phylogenic tree with human and chicken CXCL13. The degree of homology between vIL-8 and chicken CXCL13L1 at both gene (three-exon gene structure) and protein level (53.8%) is striking compared with cCXCL8 (31.1%) and other CXC chemokines **(Table 8)**.

These findings agree with our hypothesis that this virokine originated from chicken CXCL13L1 rather than cCXCL8. As human genes are the reference for nomenclature and determination of orthologues genes in other species (89). (36), we proposed here to rename vIL-8 to its orthologues, and the name should be vCXCL13.

Functional characterization of vIL-8 *in vitro* and *in vivo*

- ***In vitro***

To investigate if vIL-8 is the functional orthologue of chicken CXCL13 and binds its exclusive receptor CXCR5, I used BVES which previously optimized and used in our lab to produce biological functional recombinant proteins. However, recombinant CXCL13L1 protein expressed by BVES was not correctly folded; there are two bands on western blot (25 KDa and 15 KDa) indicated protein it may have been cleaved or digested, or there is a splice variant exists in the *SF9 cells* **(Figure 12C)**. In addition the yield was lower compared to other produced chemokines. The other proteins represent the correct size with a varying degree of degradation. To obtain correctly folded protein of chicken CXCL13s and vIL-8, I used a mammalian expression system. Transient expression systems in mammalian cells provide an attractive method for producing of recombinant proteins. The system characterized by cost affordable and good protein yields within a few days compared to other expression systems. The human embryonic kidney cells (HEK) are adapted to grow and reaching high densities even with serum-free media. HEK cells can be readily transfected using PEI, a standard transfection reagent. Besides productivity issues, the mammalian expression system maintains post-translational modifications and proper protein folding.

The recombinant CXC chemokines produce by mammalian system confirmed the presence of a band with a molecular weight of expected size corresponding to the monomeric, non-glycosylated form of cCXCL13s. Also, another faint band was present below the expected major band which most likely represents the degradation form of the chemokines **(Figure 15)**. The size of huFC and vIL-8 is higher than expected which may related to post-translation modification most probably glycosylation. The functional activity of the recombinant protein was tested by the binding assay with a positive control produced likewise and these proteins can be used for in vitro assays.

CXCL13 binds CXCR5, this interaction is required for homing of CXCR5+ B cells and T cells to lymphoid organs in human and chicken (24). CXCL13 or also known as B lymphocyte chemoattractant (BLC) binds and recruits B lymphocyte to lymphoid organs. We could first detect the expression of CXCR5 in normal naive primary bursal B cells and DT40 chicken B lymphocyte cell line. Our results of binding assay showed clearly the binding of CXCL13L1

and vIL-8 bind to CXCR5+ B cells in equivalent way, which also correlated with the expression of CXCR5 on chicken B cells. CXCL13L2 showed a slight binding with CXCR5+ B cells, however, this binding was not specific as it also binds CXCR5- HEK293 cells **(Figure 18E)**. CXCL13L3 did not show binding with CXCR5+ B **(Figure 18F)**. CXCL13L2 and L3 function in chicken is still unknown.

The previous study from our laboratories has demonstrated that vIL-8 recruits chicken B and T cells (43). B cells and activated T cells express CXCR5; the exclusive receptor for CXCL13 in human and chicken (11, 24). To determine if the binding of vIL-8 to B cells is like the cellular orthologue mediated by CXCR5, we determine with our collaborator (Dr. Sonja Härtle, Tierphysiologie, Ludwig-Maximilians-Universität, München, Germany) that both vIL-8 and CXCL13L1 specifically bind CXCR5+ clonal HEK293 human cell line, which naturally lacks this receptor. CXCL13L2 binds nonspecifically both the CXC5 positive and negative cells. Like the results of B cells binding, CXCL13L3 did not show binding with CXCR5+ clonal HEK293 cells. The results of the binding assays of the recombinant CXC chemokines and specific cCXCR5+ cells showed that vIL-8 and CXCL13L1 bind to chicken CXCR5 in the same way, and CXCR5 mediate the binding of vIL-8 with B cells. These finding confirm that vIL-8 is the functional orthologues of CXCL13L1 and binds its exclusive receptor CXCR5.

Orthologue genes perform the same function in different related species (5). In addition to the role of CXCL13 in the homing of CXCR5+ B cells and T cells to lymphoid organs, it also has a role the inflammatory process by enhancing infiltration of T and B cells to the site of infection (81). The CXCL13/CXCR5 interaction is also involved in the growth and metastasis of different types of human cancer cells (22, 40, 149), consider as targets for lymphoma diagnosis and treatment as well as a prognosis marker related to the poor prognosis (103). Activation of CXCL13/CXCR5 pathway promotes tumor growth by enhancing proliferation and inhibit apoptosis (42). Furthermore, the CXCL13/CXCR5 interaction enhances the expression of matrix metalloproteinase (MMPs), which are endopeptidases that promote tumor dissemination and metastasis (149). MDV seems to acquire vIL-8 from chicken CXCL13L1 and employs the CXCL13/CXCR5 interaction in MDV pathogenesis and tumorigenicity.

CXCL13/CXCR5 interaction elicits different intracellular signaling pathways to perform the chemokine roles. It enhances prostate cancer proliferation through c-Jun N-terminal kinases (JNKs), which is a kinase that activates the c-Jun transcription factor. Also extracellular signal-regulated kinase (ERK)1/2 mediates CXCL13/CXCR5 enhancement of cell proliferation and metastatic invasion (42). The phosphatidylinositol-4,5-bisphosphate 3-

kinase enzyme(PI3K)/ protein kinase B (PKB or Akt) is another mediator of the CXCL13/CXCR5 interaction, contribute in tumorigenicity by enhancing proliferation, invasion and maintain cell survival through anti-apoptotic effect (149).

Our findings confirm the vIL-8 interaction with CXCR5 expressed on B cells. It may also employ the CXCL13/CXCR5 intracellular signaling pathway in the tumorigenicity and metastasis in MDV, employs the CXCL13/CXCR5 pathway in MDV pathogenesis and tumorigenicity.

One should notice that a chemokine can bind to different chemokine receptors of the same family. Some leukocyte express more than one receptor, often share common ligands and perform different biological functions (15). For this, the binding of vIL-8 to other CXC chemokine receptor need further investigation.

To generate recombinant viruses which can replicate and secrete CXCL13 instead of vIL-8, we targeted different location in MDV genome. For optimal simulation of vIL-8 production and secretion, the original site is the preferred locus for insertion of chicken CXCL13. However the highly splicing region between vIL-8 with meq and other neighboring genes like RLOF4 may interfere with transcription in this region. Such interference could affect recombinant viruses pathogenicity. Beside acceptor sites in CXCL13 were predicted (http://www.fruitfly.org/cgi-bin/seq_tools/splice.pl), which may interfere with the splicing in this region. A recombinant virus of CXCL13L1 at the C-terminus of vIL-8 was generated and successfully reconstructed, however, no secreted protein was detected. For this another locus may allow virus replication and CXCL13 secretion. Selection of a location within MDV genome that permits the expression of an ectopic gene in perspective kinetics is a key for the gene function. Only genetic regions encoding non-essential genes for virus survival, and don't affect virus pathogenicity and tumor formation can be targeted. For a quite expression of chicken CXCL13s in early MDV infection; CXCL13L (1, 2, or 3) inserted under the effect of the strong immediate early cytomegalovirus promoter (pCMV) between UL45-UL46 and UL35-UL36; the insertion in unique long sites resulted in severely impaired virus replication upon reconstitution **(Figure 20)**. Previous report demonstrated that the use of immediate early strong CMV to express an ectopic gene can result in inhibition of MDV replication (110). Also, the possibility cannot be ruled out that the insertion may interfere with expression of essential adjacent genes.

Minimal fertility factor replicon (mini-F) is essential for maintaining BAC sequence replication in *E.coli*., insertion of mini-F in the non-essential US2 locus of MDV BAC allowed viral replication *in vitro* and *in vivo*, without interfering with virus pathogenicity (101, 133). The

E.coli. xanthine-guanine phosphoribosyltransferase gene (gpt) in mini-F (77) was replaced with chicken CXCL13s under the effect of CMV promoter. This site permitted pCMV-CXCL13L2 and pCMV-CXCL13L3 mutants replication and chemokines secretion. However, pCMV-CXCL13L1 mutants couldn't be reconstituted; only single infected cells have been seen after transfection and several passages. The other option is to have chicken CXCL13 driven by a weaker thymidine kinase promoter (pTK) in mini-F. The replacement of mini-F xgpt with pTK-CXCL13L (1, 2, or L3) enable recombinant viruses reconstitution and secretion of cCXCL13s **(Figure 22. C)**.

- ***In vivo***

Recombinant viruses expressing CXCL13 in mini-F under TK promoter behave *in vitro* similarly to the wild type in term of plaques formation and replication **(Figure 22).** *In vivo* experiment showed that the recombinant mutants were comparable to vIL-8 knockout viruses in their pathogenicity (26-41%), while 100% of the animals infected with wild-type virus developed the disease **(Figure 23)**. These recombinant viruses couldn't restore MDV pathogenicity *in vivo*. To quantify the secreted vIL-8 in its original location and compare it with pTK-CXCL13 in mini-F; the C-terminus of vIL-8 in the wild type virus was tagged with a 6XHis-Tag to allow detection in a comparable manner with the 6XHis-tagged chicken CXCL13s. The result showed that vIL-8 promoter enable secretion of the virokine in much higher quantity than TK promoter did. Our findings indicated that the expression level of chemokine is crucial to maintain chemokine function.

Unfortunately, all attempts to generate MDV that lacks vIL-8 but ectopically expresses comparable amount chicken CXCL13s didn't result in restoration of MDV pathogenicity. In future experiments, the synthetic CXCL13L1 gene without acceptor site could allow the insertion into the vIL-8 region, without interfering the splicing in the meq-vIL-8 region. This approach will determine if ectopic expression of CXCL13L1 results in restoration of vIL-8 function.

5. Conclusion

During the long co-evolution with chickens, MDV acquired a CXC chemokine to enhance its pathogenicity by recruiting target cells. MDV harbors two copies of a CXC chemokine named vIL-8 after the homology with the first identified chicken IL-8. The differences between MDV-vIL-8 and chicken IL-8 (cCXCL8) at the gene and protein levels, as well as the type of cells recruited request to find the real cellular orthologue for vIL-8. Orthologues genes are one particular gene in two related species acquired during evolution process. Detection of true orthologues is critical for prediction of the gene function in the related species. Furthermore, it allows the identification of the correct taxonomy. Accurate taxonomy in CXC chemokines facilitates the characterization of target receptor and function on the cells expressing the related receptor. The availability of the complete genome sequence of chicken has revealed new CXC chemokines candidates to be the true vIL-8 orthologue. Using phylogenic tree and large-scale blast-based method by orthologue detecting programs, we found that vIL-8 is the functional orthologue of chicken CXCL13L1 (also known as B lymphocyte chemoattractant (BLC) or B cell-attracting chemokine 1 (BCA-1). Gene structure and amino acid similarity between vIL-8 and cCXCL13L1 is the highest among all CXC chemokine evaluated. Furthermore, we determined the receptor of vIL-8 and found that both vIL-8 and its cellular orthologue CXCL13L1 bind CXCR5; the exclusive receptor of CXCL13. Like BLC we found that vIL-8 binds to CXCR5 positive primary chicken B cells and chicken B lymphocyte cell line chicken B cells. Our findings indicate that vIL-8 is the functional orthologue of chicken CXCL13L1, binds its receptor on the same target cells. We proposed to change the name of vIL-8 to be vCXCL13L1.

Unfortunately, all our attempts to generate a recombinant MDV that can secrete CXCL13L1 equivalent to vIL-8 have failed so far; we investigated several locations in MDV genome as well as different promoters. It was not possible to express chicken CXCL13L1 in a quantity comparable to vIL-8. Our strategy of complementing vIL-8 abrogation by ectopically expressing CXCL13L1 did not result in restoration of MDV pathogenicity. Therefore, it remains unknown if CXCL13L1 can compensate the loss of vIL-8 in MDV pathogenesis.

Further investigations are needed to identify vIL-8/CXCR5 post-receptor events and to characterize the signaling pathway. These experiments will increase our understanding of the role of this virokine in MDV pathogenesis that will help us to develop therapeutic approaches or novel vaccines.

Das Chemokin vIL8 des Virus der Marekschen Krankheit (MDV) bindet den cCXCR5 Rezeptor und ist ein funktionelles Ortholog des CXCL 13 Chemokins

Zusammenfassung

Bei dem Virus der Marek'schen Krankheit (MDV) handelt es sich um krebserzeugendes *Alphaherpesvirus* welches die Marek'sche Krankheit (MD) in Hühnern auslöst. Die Infektion von Hühnern mit virulenten Stämmen des MDV führt dabei zu hoher Mortalität, vizeralen Lymphomen, Paralyse und Immunsupression. Mit diesen Symptomen der MD geht ein hoher wirtschaftlicher Verlust einher.

Das Genom des MDV kodiert für ein CXC-Chemokin (ein chemotaktisches Zytokin) namens vIL-8, welches aufgrund von Sequenzübereinstimmungen nach dem Interleukin-8 des Huhnes (cCXCL8) benannt ist. Neuere Untersuchungen bezüglich der Funktion des vIL-8 zeigten jedoch, dass es sich nicht um ein cCXCL8-Homolog handelt. Daher ist die Identifikation eines zellulären vIL-8-Orthologs sowie Rezeptorbindungsstudien notwendig, um die Funktion des Proteins in der MDV Pathogenese zu verstehen. Im ersten Teil des Projektes wurde die evolutionäre Verwandtschaft des vIL-8 mit anderen Proteinen untersucht und eine funktionelle Charakterisierung *in vitro* vorgenommen. Phylogenetische Untersuchungen sowie Analysen der Aminosäuresequenzen von vIL-8 im Vergleich zu allen bekanten CXC-Chemokinen des Menschen und Huhns zeigten, dass es sich bei dem vIL-8 aufgrund des Fehlens eines konservierten Aminosäuresequenzmotifs (bestehend aus den Aminosäuren Glutaminsäure, Leucin und Arginin) um ein so genanntes ELR- CXC-Chemokin handelt. ELR+ CXC-Chemokine, wie das cCXCL8, binden an die Rezeptoren CXCR1 und CXCR2 und induziert die chemotaktische Anlockung von Heterophilen und Monozyten sowie die Angiogenese. Von allen untersuchten CXC-Chemokinen weißt vIL-8 die größte Ähnlichkeit mit cCXCL13L1 oder dem *B lymphocyte chemoattractant* (BLC) auf. Bei vIL-8 und cCXCL13L1 handelt es sich um ELR- CXC-Chemokine. Im Gegensatz zu der typischen Struktur von CXC-Chemokingenen mit vier Exonen, besitzen beide allerdings nur drei Exone. Unsere Untersuchungen zeigten, dass vIL-8 den cCXCR5-Rezeptor bindet (den eigentlichen Rezeptor des cCXCL13) und als chemotaktisches Zytokin für Hühner B- und T-Zellen, welche CXCR5 exprimieren, wirkt.

Um zu untersuchen ob das zelluläre cCXCL13 das vIL-8 in der MDV-Pathogenese und Tumorentstehung funktionell ersetzen kann, erzeugten wir, basierend auf dem *bacterial artificial chromosome* (BAC) des hochvirulenten RB1B-Stamms, verschiedene rekombinante Virusmutanten. Um dabei die Produktion und Sekretion von vIL-8 nachzuahmen, wurde

cCXCL13 in die folgenden Genombereiche eines vIL-8 negativen BACs inseriert: 1) in der 3' Region des ursprünglichen vIL-8, in welcher extensives Splicing die Sekretion jedoch inhibierte; 2) innerhalb der UL-Region (auf die Gene UL35 and UL45 folgend), wodurch es zu einer massiven Beeinträchtigung des viralen Wachstums kam; 3) innerhalb des nicht-essentiellen mini-F Lokus unter der Kontrolle des starken Promotors des Cytomegaloviruses, wodurch es ebenfalls zur Beeinträchtigung des Wachstums kam, 4) innerhalb des mini-F Lokus unter der Kontrolle des schwächeren Thymidinkinasepromotors. Diese Virusmutante ermöglichte die Sekretion des Proteins und erlaubte eine vergleichbare Replikation *in vitro* und *in vivo*. Des Weiteren zeigte sich, dass die Funktion des vIL-8 bezüglich der Pathogenese in vivo durch das cCXCL13 nicht kompensiert werden konnte. Die Gründe hierfür sind nicht bekannt, könnten jedoch mit der Position des Gens innerhalb der mini-F sowie der Kontrolle durch den fremden Promotor im Zusammenhang stehen. Um dieser Frage nachzugehen und die Sekretion des vIL-8 im Wildtypvirus zu untersuchen, wurde ein Polyhistidin-Tag (6xHis-tag) an das c-terminale Ende des Gens fusioniert. Unsere Ergebnisse zeigten, dass das Expression des vIL-8 unter Kontrollen des eigenen Promotors viel höher als die des cCXCL13 unter Kontrolle des TK-Promotors war. Dieser Umstand könnte erklären warum das cCXCL13 nicht in der Lage war, dass vIL-8 in der MDV-Pathogenese zu kompensieren.

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Declaration

I hereby declare that the work presented in this thesis has been conducted by my self, and are suitably referenced and acknowledged. I further declare that this thesis has not been submitted before, either in the same or a different form, to this or any other university for a degree.

Berlin, 05.08.2016

Ibrahim Alzuheir